Detachment of Cultured Cells from the Substratum Induced by the Neutrophil-derived Oxidant NH_2Cl: Synergistic Role of Phosphotyrosine and Intracellular Ca^{2+} Concentration

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Abstract. The neutrophil-derived, membrane-permeating oxidant, NH_2Cl, (but not the non–membrane-permeating chloramine, taurine-NHCl) induced detachment of fetal mouse cardiac myocytes and other cell types (fibroblasts, epithelial cells, and endothelial cells) from the culture dish, concomitant with cell shrinkage ("peeling off"). Stimulated human neutrophils also induced peeling off of cultured mouse cardiac myocytes when the latter were pretreated with inhibitors of ·OH and elastase. Immunofluorescence microscopy revealed that the NH_2Cl-induced peeling off of WI-38 fibroblasts is accompanied by disorganization of integrin $\alpha_5\beta_1$, vinculin, stress fibers, and phosphotyrosine (p-Tyr)-containing proteins. Decrease in the content of the p-Tyr–containing proteins of the NH_2Cl-treated cells was analyzed by immunoblotting techniques. Coating of fibronectin on the culture dish prevented both NH_2Cl-induced peeling off and a decrease in p-Tyr content. Preincubation with a protein–tyrosine phosphatase inhibitor, sodium orthovanadate (Na_3VO_4), also prevented NH_2Cl-induced peeling off, suggesting that dephosphorylation of p-Tyr is necessary for peeling off. NH_2Cl-induced peeling off was accompanied by an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in mouse cardiac myocytes and WI-38 fibroblasts. The absence of extracellular Ca^{2+} prevented both NH_2Cl-induced peeling off and increased $[Ca^{2+}]_i$, both of which did occur on subsequent incubation of the cells in Ca^{2+}-containing medium. These observations suggest that an increase in $[Ca^{2+}]_i$ is also necessary for peeling off. Depletion of microsomal and cytosolic Ca^{2+} by incubation with the microsomal Ca^{2+}-ATPase inhibitor 2′,5′-di(*tert*-butyl)-1,4-benzohydroquinone (BHQ) plus EGTA prevented both NH_2Cl-induced increases in $[Ca^{2+}]_i$ and peeling off. Direct inhibition of microsomal Ca^{2+} pump activity by NH_2Cl may participate in the NH_2Cl-induced $[Ca^{2+}]_i$ increment. A combination of p-Tyr dephosphorylation by genistein (an inhibitor of tyrosine kinase) and an increase in $[Ca^{2+}]_i$ by BHQ could also induce peeling off. All these observations suggest a synergism between p-Tyr dephosphorylation and increased $[Ca^{2+}]_i$ in NH_2Cl-induced peeling off.

CELLULAR adhesion to the extracellular matrix (ECM)[1] is a critical step in development and differentiation, metastasis, and normal cell growth (21). This adhesion is mediated by surface-bound receptors of the integrin superfamily (α/β heterodimers) (24). Integrin-mediated linkage of the ECM to the cytoskeleton occurs at focal adhesion sites, specialized regions where the ventral cell surface and substratum are most closely apposed and tightly linked (58). Focal adhesions consist of clustered integrins (4, 8, 24) linked to stress fibers (actin cables) (4, 58) by cytoplasmic plaque-containing proteins such as vinculin, paxillin, and talin (4). These cytoskeletal associations participate in the adhesive process by stabilizing integrin–ligand interactions (8) and supporting cell shape (58).

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1.*Abbreviations used in this paper*: BHQ, 2′,5′-di (*tert*-butyl)-1,4-benzohydroquinone; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DMTU, dimethylthiourea; ECM, extracellular matrix; Fn, fibronectin; HOCl, hypochlorous acid; pNPP, *p*-nitrophenyl phosphate; PTPase, protein-tyrosine phosphatase; p-Tyr, phosphotyrosine.

Much evidence suggests that neutrophil-generated oxidants injure several tissues at sites of inflammation (10, 20) and cardiac tissue at ischemic sites during the early hours of reperfusion (9, 40, 45). A common feature of neutrophil-induced tissue injury is detachment of endothelial and epithelial cells from the basement membrane or ECM (10, 22), and this may be due to neutrophil-derived oxidants (22, 23, 43, 61).

When stimulated, neutrophils, which possess NADPH oxidase and myeloperoxidase, undergo a respiratory burst during which oxygen is reduced to superoxide anion ($\cdot O_2^-$) by NADPH oxidase. $\cdot O_2^-$ initiates a series of reactions that produce toxic oxidizing species and dismutates rapidly to yield hydrogen peroxide (H_2O_2). H_2O_2 is reduced to highly reactive hydroxyl radicals ($\cdot$OH) via the iron-mediated Fenton reaction, which has been reported to be a trigger for lipid peroxidation (37). Myeloperoxidase, on the other hand, catalyzes the oxidation of Cl^- by H_2O_2 to yield hypochlorous acid (HOCl). Most of the highly reactive HOCl combines nonenzymatically with nitrogenous compounds such as ammonia and taurine to generate long-lived oxidants, inducing monochloramines such as NH_2Cl (membrane permeating) and taurine-NHCl (non–membrane permeating) (55).

We reported previously that addition of HOCl or NH_2Cl to the myocytes caused remarkable morphological changes manifested by the detachment of some peripheral cells from the culture dish without balloon formation and hypercontraction (41) (peeling off). In contrast, xanthine + xanthine oxidase (a source of $\cdot O_2^-$, H_2O_2, and $\cdot$OH) or H_2O_2 (a source of $\cdot$OH) caused hypercontraction concomitant with balloon formation of the cells without peeling off (41). NH_2Cl-induced peeling off was prevented by methionine (an HOCl scavenger), but not by superoxide dismutase, catalase, deferoxamine (an iron chelator), or dimethylthiourea (DMTU) (an $\cdot$OH scavenger), all of which could prevent the xanthine + xanthine oxidase– and H_2O_2-induced impairments (41), indicating that the mechanism of NH_2Cl-induced peeling off is different from that of xanthine + xanthine oxidase– or H_2O_2-induced impairment, and that intracellularly produced $\cdot$OH may participate in the induction of hypercontraction. We speculated that some of the proteins that contribute to cell–substratum adhesion, such as ECM, focal adhesion proteins, and/or the cytoskeleton, may have been damaged by NH_2Cl.

Focal adhesions are considered not only to have a structural function, but also to serve as sites for initiating signal transduction from the ECM to the cell interior. Potential intracellular messengers include protein–tyrosine phosphorylation (6, 24) and Ca^{2+}-dependent protein kinase C (62). Focal adhesions contain protein tyrosine kinases such as $pp60^{V\text{-}SRC}$ (44) and $pp125^{FAK}$ (focal adhesion kinase) (6, 18, 24, 29, 47) localized to the residual focal adhesions of cells transformed by Rous sarcoma virus (44) and normal cells, respectively, and activated by cell–substratum adhesion (6, 16, 18, 29) and integrin clustering (29, 31). An increase in protein–tyrosine phosphorylation has been observed during the attachment of cells to the substratum (6, 17, 29). It has been recently reported that endothelial cell attachment and spreading on fibronectin (Fn) or vitronectin resulted in an elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (48). The relationships between dephosphorylation of phosphotyrosine (p-Tyr) and cell detachment from the substratum, however, or between $[Ca^{2+}]_i$ and cell–substratum detachment have not been studied in detail. The mechanism of neutrophil-induced cellular injuries, that is, which particular cytotoxic mediators produced by stimulated neutrophils induce which particular type(s) of injury, is also unclear.

In this work, we studied the cellular and molecular mechanisms of NH_2Cl-induced peeling off using various kinds of cultured cells, including cardiac myocytes. We also studied the correlation between neutrophil-induced morphological impairment and NH_2Cl-induced peeling off. Our aims were to clarify (*a*) whether NH_2Cl-induced peeling off would occur in other kinds of cells, (*b*) whether and how peeling off could be prevented, (*c*) the possible mechanisms of NH_2Cl-induced peeling off, and (*d*) whether stimulated neutrophils could induce peeling off of cells to which they were adhered.

Materials and Methods

Agents

Antibodies. Rabbit anti–human Fn receptor polyclonal antibody and rhodamine (TRITC)-conjugated goat anti–rabbit and anti-mouse IgG were obtained from Chemicon International Inc., Temecula, CA; mouse anti–human vinculin mAb was from Serotec Ltd., Oxford, UK; and mouse anti–p-Tyr mAb PY20 and HRP-labeled protein A were from ICN Biochemicals, Inc., Costa Mesa, CA. FITC-conjugated phalloidin was from Sigma Chemical Co., St Louis, MO.

ECM Proteins. Bovine plasma Fn was obtained from Itoham Foods Inc., Hyogo, Japan; rat laminin was from Chemicon International Inc.; collagen types I, III, and IV were from Wako Chemical Co., Osaka, Japan.

Chemicals. NaOCl, NH_4Cl, taurine, BSA, orthovanadate (Na_3VO_4), bepridil hydrochloride, amiloride, 2′,5′-di (*tert*-butyl)-1,4-benzohydroquinone (BHQ), colcemid, paraformaldehyde, Triton X-100, glycerol, PMA, dimethylthiourea (DMTU), deferoxamine, and methionine were purchased from Sigma Chemical Co. Aprotinin, leupeptin, pepstatin, antipine, and PMSF were from Boehringer-Manheim GmbH, Mannheim, Germany. MEM, methionine-free MEM, digitonin, *p*-phenylenediamine dihydrochloride, *p*-nitrophenyl phosphate (pNPP), genistein, and verapamil hydrochloride were obtained from Wako Chemical Co.; FBS was from Flow Laboratories Inc., North Ryde, N. S. W., Australia. Trypsin (1:250) was from Difco Laboratories, Inc. Detroit, MI. Formaldehyde solution (EM grade) was from Electron Microscopy Sciences, Fort Washington, PA. The acetoxymethyl ester of fluo-3 was obtained from Dojindo Laboratories, Kumamoto, Japan. Cytochalasin D was from Aldrich Chemical Co., Milwaukee, WI. Nifedipine, sodium cacodylate, *S*-collidine, osmium tetraoxide solution, and lead citrate were obtained from Nacalai Tesque Inc., Kyoto, Japan. α_1-antitrypsin (human plasma) was from Calbiochem Corp., La Jolla, CA. Human recombinant tumor necrosis factor-α was from Genzyme Corp., Cambridge, MA, and platelet activating factor (C18) was obtained from Bachem Feinchemikalien AG, Bubendorf, Switzerland. Uranyl acetate was from Fluka Chemika-BioChemica, Tokyo, Japan. $^{45}CaCl_2$ was purchased from New England Nuclear, Boston, MA.

NH_2Cl and taurine-NHCl were synthesized as previously described (55) by adding 0.1 M NaOCl in 0.01 M NaOH to a solution of N-compounds (NH_4Cl for NH_2Cl or taurine for taurine-NHCl) at 4°C. A 10:1 ratio of N-moiety to NaOCl was used to prevent formation of dichloramines. Their respective concentrations were determined by assuming a molar extinction coefficient of 429 M/cm at 242 mm.

The following solutions were prepared in our laboratory: FBS-MEM (MEM supplemented with 10% [vol/vol] FBS); BSS containing (mM) NaCl 116, KCl 5.4, $CaCl_2$ 1.8, $MgSO_4$ 1.0, glucose 5.6, and Hepes 10.0, pH 7.3, adjusted with NaOH; Ca^{2+}-free BSS containing (mM) NaCl 116, KCl 5.4, $MgSO_4$ 3.0, EGTA 2.0, glucose 5.6, and Hepes 10.0, pH 7.3 adjusted with NaOH; high K^+ solution containing (mM) NaCl 5.4, KCl 116, $CaCl_2$ 1.8, $MgSO_4$ 1.0, glucose 5.6, and Hepes 10.0, pH 7.3, adjusted with KOH; and Ca^{2+}-free Mn^{2+} solution containing (mM) NaCl 5.4, KCl 116, $MgSO_4$ 1.0, $MnCl_2$ 1.8, glucose 5.6, and Hepes 10.0, pH 7.3, adjusted with KOH; PHEM buffer containing (mM) Pipes 60.0, Hepes 25.0, EGTA, 10.0,

$MgCl_2$ 2.0, pH 6.1, and 6.9, adjusted with NaOH; lysis buffer containing (mM) 2-glycerophosphate 60, paranitrophenol 20, Na_3VO_4 0.5, NaCl 250, $MgCl_2$ 15, Hepes 40, and 1% Triton X-100, pH 7.5, adjusted with NaOH.

Cell Culture

To prepare mouse cardiac myocytes and fibroblasts, mouse heart ventricles were removed from 14-d-old fetuses (ICR strain) and dissociated into single isolated cells by trypsinization, as described previously (41, 42). To obtain separate myocyte and fibroblast cultures, the cells were plated at 37°C for 1 h in FBS-MEM. This caused most of the fibroblasts to adhere to the culture dish surface. These adhered cells were grown in FBS-MEM under a water-saturated atmosphere of 5% CO_2 in air. The remaining, unadhered cells (mostly myocytes) were collected and passed through a filter (Swinex; Millipore Corp., Bedford, MA) lined with lens paper, plated (2×10^5 cells per dish) on plastic petri dishes (3001; Falcon Labware, Becton Dickinson & Co., Lincoln Park, NJ) or on glass coverslips in petri dishes, and incubated in FBS-MEM under the conditions described above. To obtain a large cell sheet in the center of the dish, the dishes were subjected to gyratory shaking for 30–60 s immediately after plating. After 1 d, almost all the myocytes became attached to and spread on the surface of the coverslips and petri dishes, and all the myocytes in each sheet beat spontaneously and synchronously.

Human umbilical vein endothelial cells prepared by Morinaga Institute of Biological Science (Yokohama, Japan) were plated on type I collagen–coated dishes and cultured in FBS-MEM.

Fibroblast-like cell lines 3Y1-B clone 1–6 (derived from whole fetal rat), L (derived from mouse connective tissue), WI-38 (derived from human lung), and MRC (derived from human lung); and epithelial-like cell lines KB (derived from human epidermoid carcinoma) and MDBK (derived from bovine kidney) were obtained from Riken Cell Bank (Saitama, Japan). The cells were grown as monolayers in square flasks (3013; Falcon Labware) at 37°C in FBS-MEM and dispersed as single isolated cells by trypsinization 1 d before use, plated on glass coverslips, and cultured in FBS-MEM.

NH_2Cl Treatment

20 h after cultivation, the cells were washed in PBS and preincubated in methionine-free MEM (to avoid any potential antioxidative effect of methionine and serum) or Ca^{2+}-free or Ca^{2+}-containing BSS for 10 s to 20 min at 37°C, and then they were exposed to 15-100 μM NH_2Cl for 2–30 min at 37°C in the same solution. Cell morphology was observed using an inverted phase-contrast microscope (IMT-2; Olympus Corp., Precision Instruments Division, Tokyo, Japan; or Diaphot; Nikon Instrument Group, Tokyo, Japan) while the cells were maintained at 37°C and pH 7.3, and gas composed of 5% CO_2 and 95% air flowed into the chamber.

Preparation and Addition of Neutrophils to Cultured Cardiac Myocytes

Polymorphonuclear leukocytes (neutrophils) were separated from heparinized peripheral blood from healthy donors by a previously published Percoll gradient centrifugation protocol, with a minor modification (30). Briefly, after removal of the platelet-rich plasma, the buffy coat was layered on a 55% isotonic Percoll solution and centrifuged at 500 *g* for 20 min. The leukocyte layer overlying the erythrocyte pellet was collected. Contaminated erythrocytes were lysed with 0.15 M NH_4Cl. These preparations comprised >90% neutrophils and >98% viable cells by the erythrosine B exclusion test. The neutrophils were suspended in HBSS at a concentration of 10^7 cells/ml.

Trypsin-dissociated fetal mouse cardiac myocytes were plated (10^5 cells per dish) on glass coverslips in petri dishes without gyratory shaking, and cultured in FBS-MEM at 37°C. 20 h after cultivation, the coverslips contained small cell sheets consisting of 5–15 synchronously beating cardiac myocytes. After the incubation medium was changed to HBSS, the myocytes were pretreated with a noncytotoxic concentration of platelet activation factor (10^{-6} M) for 30 min (this treatment has been reported to increase the effects of neutrophils [27]). Human neutrophils (2×10^6 cells per dish) were layered onto the cardiac myocytes, and coincubation for 30 min permitted adherence of the two cell types. PMA (100 ng/ml) and tumor necrosis factor-α (10 ng/ml)(27) were then applied to stimulate the neutrophils. In some experiments, cardiac myocytes were pretreated with deferoxamine (10 mM) for 6 h in FBS-MEM, and then incubated in HBSS on changing the medium from FBS-MEM to HBSS. In other experiments, DMTU (10 mM), α_1-antitrypsin (50 μg/ml), and/or methionine (10 mM) were spread on the myocytes 30 min before the addition of the neutrophils in HBSS. All of these inhibitors and platelet activation factor were present in the medium throughout the neutrophil-treatment experiments. In all experiments, neutrophil-induced morphological injury was assessed at 2.5–3 h after the addition of the neutrophils using an inverted phase-contrast microscope (Olympus IMT-2 or Nikon Diaphot).

Coating of ECM Proteins

Glass coverslips (25 × 25 mm) in petri dishes were overlaid with 300 μl Fn (2.5–20 μg/ml), laminin, and/or collagen types I, III, and/or IV (20–40 μg/ml each) and incubated overnight at 4°C. After blocking with 1% BSA for 1 h at 37°C and washing with FBS-MEM, mouse cardiac myocytes or WI-38 cells were plated on the coverslips and cultivated as described above. All these coverslips were sterilized by exposure to UV light for 10 min after being overlaid with the ECM proteins.

To study the effect of NH_2Cl-treated Fn, 20 μg/ml Fn was treated with NH_2Cl at a final concentration of 300 μM for 30 min at 37°C in a plastic test tube before being overlaid on glass coverslips as described above.

Measurement of Extent of Peeling Off

Cells were photomicrographed before and after NH_2Cl treatment; the photographs were enlarged, and the areas showing attached cells were cut out and weighed. The percent of cells remaining attached to the substratum was calculated by dividing the weight of the area of attached cells after treatment by the weight of the area of attached cells before treatment × 100.

Electron Microscopy

Peeled-off cardiac myocytes identified by phase-contrast microscopy were prepared for transmission electron microscopy. The location of the cells of interest was marked with a marker pen on the outside of the culture dishes with the aid of an inverted phase-contrast microscope. The culture dishes with the cells were washed with PBS to remove the reaction medium. The cells were fixed for 2 h at 4°C with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). They were then rinsed with the same buffer at 4°C, postfixed in 1% osmium tetraoxide solution in 0.1 M *S*-collidine buffer (pH 7.3) for 1 h at 4°C, and rinsed in distilled water at 4°C. Next, they were stained at 4°C for 1 h with 1% uranyl acetate in distilled water and rinsed with distilled water at 4°C. After dehydration by passage through a series of graded concentrations of ethanol, the cells were embedded in Epon. The polymerized Epon containing the cells was detached from the culture dishes after brief immersion in liquid nitrogen. After verifying the target cells using both phase-contrast and stereoscopic microscopy followed by marking their location with a marker pen on the side of the Epon opposite the cells, a small piece of Epon containing the cells was prepared. Thin sections of the Epon piece were cut parallel to the basement membrane with an ultramicrotome (Ultracut E; Reichert-Jung, Wien, Austria), sequentially stained with uranyl acetate and lead citrate, and examined by electron microscopy (JEM-2000EX II; JEOL, Tokyo, Japan).

Polymerization of Actin

Actin was extracted for 30 min at 2°C from an acetone powder of rabbit skeletal muscle, clarified for 2 h at 100,000 *g*, and polymerized in 0.1 M KCl, following Mommaerts' procedure (39). Treatment with 0.6 M KCl was carried out as described by Spudich and Watt (50). The depolymerized actin was finally clarified by centrifugation for 3 h at 100,000 *g*. Polymerization of F-actin was effected by adding KCl to a final concentration of 0.1 M. The degree of polymerization was always checked by measuring the flow birefringence at 1,000 s^{-1} (36), and highly polymerizable samples were used for the experiments. Protein concentration was determined by a Biuret reaction using a serum albumin standard.

Viscometry

To determine specific viscosity, Fn (20 μg/ml) was treated with PBS or 300 μM NH_2Cl for 30 min at 37°C in test tubes, and polymerized actin (F-actin, 2 mg/ml) was treated with PBS, 100 μM NH_2Cl, or 10 μM cytochalasin D for 20 min at 37°C. Viscometry was performed using an Ostwald-type viscometer (Iwaki Glass Co., Chiba, Japan) (11) of 0.3-ml capacity with an outflow time of ~8 s for water at 25°C. Specific viscosity was defined in a

steady-state condition as flow time of sample solution divided by flow time of the corresponding buffer minus 1.

Immunofluorescent Microscopy

WI-38 cells grown overnight on glass coverslips were treated with or without NH_2Cl in Ca^{2+}-containing or Ca^{2+}-free BSS and rinsed with PBS. For the staining of $\alpha_5\beta_1$ integrin, vinculin, or p-Tyr, the cells on the coverslips were fixed with 3% paraformaldehyde in PBS containing 2% sucrose for 30 min, permeabilized with 0.1% Triton X-100 in PBS for 30 min, and incubated in 0.15 M glycine in PBS for 30 min at room temperature. They were preincubated with the blocking solution (1% BSA in PBS) for 1 h and then incubated with a primary antibody (anti-Fn receptor [1:100 dilution], anti-vinculin [1:10 dilution], or anti-p-Tyr [1:100 dilution] in blocking solution for 1 h at 37°C. After washing with PBS for 1 h at room temperature, the secondary antibody (rhodamine [TRITC]-conjugated goat anti–rabbit [against Fn receptor antibody] or rhodamine [TRITC]-conjugated goat anti–mouse Ig [against anti-vinculin and anti-p-Tyr antibodies]) in blocking solution was applied for 1 h at 37°C in the dark, washed extensively in PBS for 3 h at 4°C, and then the stained cells were mounted in 90% glycerol in PBS containing 1 mg/ml *p*-phenylenediamine.

For the staining of stress fibers, the cells were treated with 1% formaldehyde in Pipes, Hepes, EGTA, and Mg (PHEM) buffer (pH 6.1) containing 0.36% Triton X-100 for 2 min. This was followed by a 10-min treatment with 3.2% formaldehyde in PHEM buffer (pH 6.1) containing 0.5% Triton X-100 at room temperature. The cell-covered coverslips were then rinsed twice with the PHEM buffer (pH 6.9) and stained for 25 min with FITC-conjugated phalloidine (330 nM) in PHEM buffer (pH 6.9) at room temperature in the dark. Then they were washed extensively with PHEM buffer (pH 6.9) and mounted as described above. Stained cells were viewed by fluorescence microscopy (Vanox-T; Olympus Corp., Precision Instruments Division).

SDS-PAGE and Western Blot Analysis

WI-38 cells grown confluently in 60-mm plastic petri dishes were treated with or without 50 μM NH_2Cl for 10 min in Ca^{2+}-containing or Ca^{2+}-free BSS. After rinsing with TBS, the cells were lysed by scraping them with a rubber policeman into 200 μl lysis buffer containing 1 μg/ml protease inhibitors (aprotinin, leupeptin, pepstatin, antipine, and PMSF). The lysates were set on ice for 10 min and then centrifuged at 15,000 rpm for 10 min at 4°C. Sample buffer with (for vinculin and p-Tyr) or without (for integrin $\alpha_5\beta_1$) β-mercaptoethanol was added to each supernatant, and then the supernatants were boiled for 5 min at 100°C. Protein concentrations were determined by protein assay (BIO-Rad Laboratories, Hercules, CA). Samples containing equal amounts of protein were electrophoresed on 7.5% SDS–polyacrylamide gels and then were either stained with Coomassie blue or transferred to nitrocellulose. After blocking of the nitrocellulose with blocking solution (3% BSA in PBS), the samples were incubated with a primary antibody (anti-Fn receptor [1:1,000 dilution], anti-vinculin [1:100 dilution] or anti-p-Tyr [1:1,000 dilution] in blocking solution for 2 h at room temperature. After washing in the blocking solution for 30 min, they were incubated with HRP-labeled protein A (against anti-Fn receptor antibody, 1:2,000 dilution) or HRP-labeled anti–mouse Ig (against anti-vinculin or anti-p-Tyr antibodies, 1:2,000 dilution) in blocking solution for 2 h at room temperature. They were then washed in TBS for 30 min and analyzed with an enhanced chemiluminescence detection kit (Amersham Corp., Arlington Heights, IL).

Measurement of Protein–Tyrosine Phosphatase Activity

WI-38 fibroblasts were untreated or treated with 50 μM NH_2Cl for 2 min at 37°C (the time at which peeling off was most severe) in Ca^{2+}-containing BSS. Then the supernatants were decanted, washed once with PBS, and lysed in 1 ml of ice-cold lysis buffer (RIPA buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, and 10% glycerol) containing 10 μg/ml aprotinin, 1 mM PMSM, and 10 μg/ml leupeptin. The lysates were centrifuged at 10,000 *g* for 15 min at 4°C and the resultant supernatants were used in the phosphatase assay. The assay mixture (200 μl) containing 40 mM MES (pH 5.0), 1.6 mM DTT, 25 mM pNPP, and an equal amount of protein from each sample was incubated at 30°C for 30 min. The reaction was terminated by the addition of 200 μl of 1 N NaOH and the absorbance at 410 nm was determined (56). The assay of the protein–tyrosine phosphatase (PTPase) activity of cell lysates was also performed using ^{32}P-labeled synthetic peptide Raytide (51). The assay mixtures (100 μl) containing cell lysates, 25 mM imidazole (pH 7.2), 0.1 mg/ml BSA, 10 mM DTT, and 1 μl of ^{32}P-labeled Raytide (~10,000 cpm/ml) were incubated at 30°C for 30 min. Labeling of Raytide with $pp60^{v\text{-}src}$ kinase was performed as described previously (51, 56). The reaction was terminated by the addition of 0.75 ml of an acidic charcoal mixture containing 0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM NaH_2PO_4 and 4% Norit A (Fisher Scientific Co., Fair Lawn, NJ). After centrifugation in a microfuge, the amount of radioactivity in 0.5 ml of supernatant was measured by a liquid scintillation counter.

Measurement of $[Ca^{2+}]_i$ Changes

To measure changes in $[Ca^{2+}]_i$, cultured cardiac myocytes and WI-38 fibroblasts were exposed to 0.5 and 5 μM, respectively, of the acetoxymethyl ester of fluo-3 (25) in 2 ml FBS-MEM at 37°C for 30 min. Loading with fluo-3 did not appreciably affect the spontaneous beating rate of cardiac myocytes. The cells were washed with 10 ml BSS and incubated at 37°C for 1–2 h in 2 ml fresh BSS. Each culture dish was mounted on the microscope stage and superfused constantly with Ca^{2+}-containing or Ca^{2+}-free BSS at 37°C at a flow rate of 2 ml/min, which exchanged the medium bathing the cells every 4–5 s. A 1-mm-thick acrylic disk with a 10 × 10 mm center opening and inlet and exit ports was set stably in the dish. An excitation wavelength of 450–490 nm was obtained using a 100-W xenon lamp with interference filters (EX 450-490; Nikon Inc. Instrument Group). The fluorescence light was collected with a ×40 objective lens (ELWD 160/0-2.5; Nikon Inc. Instrument Group) and interference filters (BA 520; Nikon Inc. Instrument Group), which transmitted the fluorescence emission at >520 nm. The fluorescence signal was detected using a P1 system (Nikon Inc. Instrument Group). Fluorescence was measured using fields containing 3–10 cells.

To minimize any toxic effects of fluorescence, the microscope was shut off frequently, and the beating and morphology of the cells were observed using the phase-contrast system during the shutoff period of the light-pass of the fluorescence system.

Estimation of Cytosolic-free Calcium Concentrations from Fluorescence Signals

Calibration of the fluorescence signal was achieved using the method described for fluo-3, with some modifications (25). The cardiac myocytes and WI-38 fibroblasts were superfused with a high K^+ solution, which allowed calcium to enter the cells and saturate the fluo-3 to give maximum fluorescence (F_{max}); this usually was attained within 1 min and sustained for ~10 s. The cells were then exposed to Ca^{2+}-free Mn^{2+} solution; saturation with Mn^{2+} reduces the F_{max} of fluo-3 to ~20% of that of the Ca^{2+}-saturated dye. Thus, the predicted F_{max} would be $(F_{Mn} - F_{bkg})/0.2 + F_{bkg}$, where F_{Mn} represents the fluorescence measured in the Mn^{2+} solution and F_{bkg} represents the background signal produced by the photomultiplier's dark-current and residual autofluorescence from the optics and cell remnants. After the fluorescence intensity of the cells stabilized in the Mn^{2+}solution, which took ~3 min, the cells were lysed with 40 mM digitonin to release the dye and enable the F_{bkg} to be recorded. Since the fluorescence of metal-free fluo-3 is 1/40 that of the Ca^{2+} complex, the minimum fluorescence (F_{min}) (fluo-3 fluorescence in the absence of Ca^{2+}) is given by the equation $F_{min} = (F_{max} - F_{bkg})/40 + F_{bkg}$. $[Ca^{2+}]_i$ could then be estimated using the equation $[Ca^{2+}]_i = K_d (F - F_{min})/(F_{max} - F)$, where F is the cellular fluorescence and K_d is 400 nM at physiological ionic strength (25).

Measurement of ATP-dependent $^{45}Ca^{2+}$ Uptake in and Release from Sarcoplasmic Reticulum Vesicles

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle, and ATP-dependent $^{45}Ca^{2+}$ uptake activity was measured as described previously (49). Vesicles (10 mg/ml) were incubated in a medium containing 20 mM MOPS/KOH (pH 7.0), 0.1 M KCl, 5 mM $MgCl_2$, and 0.1 mM $^{45}CaCl_2$ (20 KBq/ml) in the absence or presence of various concentrations of NH_2Cl, and a $^{45}Ca^{2+}$ uptake reaction was started at 25°C by addition of ATP (1 mM final). $^{45}CaCl_2$ release from sarcoplasmic reticulum vesicles was measured as described previously (38). Vesicles (4 mg/ml) were passively loaded at 0°C for 24 h with 1 mM $^{45}CaCl_2$ (1 MBq/ml) in 20 mM MOPS/KOH (pH 7.0) and 0.1 M KCl. A 5-ml aliquot of vesicles was diluted into a medium (500 ml) containing 20 mM MOPS/KOH (pH 7.0), 0.1 M KCl, 1 mM EGTA, 10 mM $MgCl_2$, and 10 μM ruthenium red in the presence or absence of NH_2Cl. $MgCl_2$ and ruthenium red were included to inhibit the Ca^{2+} release channels. Vesicles in the reaction mixture were fil-

tered through a 0.45-mm filter (Millipore Corp., Bedford, MA), washed with the ice-cold solution containing 20 mM MOPS/KOH (pH 7.0), 0.1 M KCl, and 5 mM $LaCl_3$, and the $^{45}Ca^{2+}$ radioactivity remaining in the filter was counted.

Statistical Analysis

All values are expressed as mean ± SEM. Comparisons between two means were performed by Student's t test. Differences at $P < 0.01$ were considered significant.

Results

NH_2Cl-induced Peeling Off

Fig. 1 shows NH_2Cl-induced morphological changes in various kinds of cells observed using an inverted phase-contrast microscope. When the cardiac myocytes were exposed to 100 μM NH_2Cl in methionine-free MEM, all the cells in a sheet suddenly ceased spontaneous beating, and some cells, in particular those at the periphery of the sheets, detached from the culture dish concomitant with cell shrinkage (peeling off) (Fig. 1 *B*). At 37°C, this began within 10 s, and the peeling-off area gradually increased during 5 min of further incubation with NH_2Cl. Although cells detached from the substratum, cell-to-cell adhesion was not affected. No change, however, was observed at temperatures lower than 15°C.

NH_2Cl-induced peeling off was also observed in all the other kinds of cells we investigated, including fibroblast-like cells such as WI-38 cells (Fig. 1 *D*), mouse heart fibroblasts, 3Y1-B clone 1-6, MRC-5, and L cells (data not shown), epithelial cells such as MDBK (Fig. 1 *F*) and KB (data not shown) cells, and human endothelial cells (Fig. 1 *H*). Another NH_2Cl-induced morphological change was that some peripheral regions of individual cells remained on the substratum as thin fibers (Fig. 1, *D*, *F*, and *H*; *arrows*). More than 90% of these cells excluded vital dyes (erythrosin B or trypan blue) for 1 h after exposure to NH_2Cl, indicating that the NH_2Cl-induced cell detachment was not due to cell death. Exposure to higher concentrations (>100 μM) of NH_2Cl for >2 h, however, did cause cell death. Fibroblasts and endothelial cells were more susceptible than cardiac myocytes to NH_2Cl toxicity. When the incubation medium was changed to FBS-MEM after treatment with low concentrations (≤50 μM) of NH_2Cl for 1 h, all cell types readhered to and spread on the substratum within 24 h and resumed cell growth; cardiac myocytes resumed spontaneous beating as well.

No morphological damage to nuclei, mitochondria, or myofibrils in NH_2Cl-treated myocytes was evident by electron microscopy (Fig. 2 *B*). We could not know, however, whether and how cellular membranes, especially in the region of focal adhesions, were altered because the thin sections were cut parallel to the basement membrane, not perpendicular to it, which would have permitted visualization of the focal contacts of the cells.

In contrast to membrane-permeating NH_2Cl, a potent oxidizing but non–membrane-permeating chloramine, taurine-NHCl (15), did not cause peeling off of any of the cell types (data not shown).

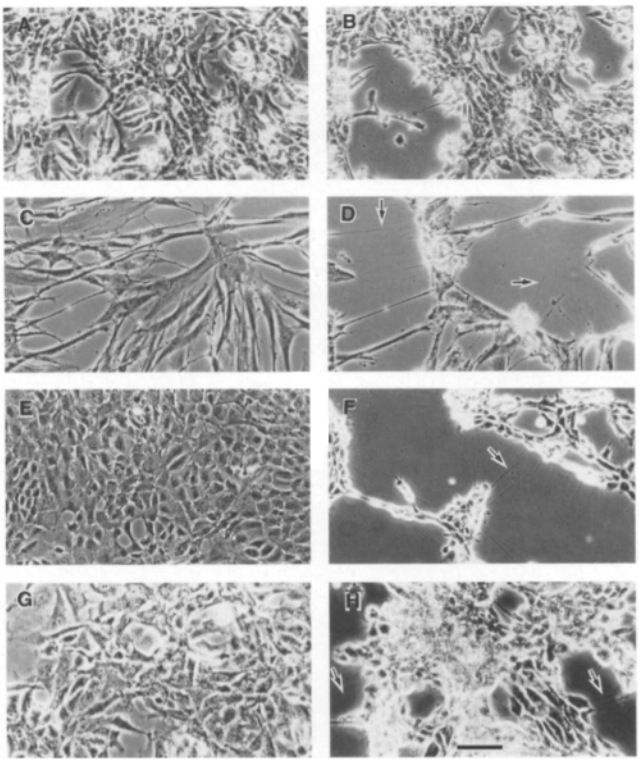

Figure 1. Phase-contrast microscopy showing the effect of NH_2Cl on the morphology of several kinds of cells. Cells were cultivated on glass coverslips for 20 h at 37°C in FBS-MEM and then exposed to 100 μM (cardiac myocytes) or 50 μM (other cells) NH_2Cl for 10 min at 37°C in methionine-free MEM. (A–H) The cell sheet before (*A*, *C*, *E*, and *G*) and after (*B*, *D*, *F*, and *H*) NH_2Cl treatment of mouse cardiac myocytes (*A* and *B*), WI-38 human lung fibroblasts (*C* and *D*), MDBK bovine kidney epithelial cells (*E* and *F*), and human endothelial cells (*G* and *H*). Arrows indicate the thin fiberlike structures of parts of peripheral regions of individual cells that remained on the substratum after treatment with NH_2Cl. Bar, 50 μm.

Effect of SH Group Oxidizing and Reducing Compounds

NH_2Cl oxidizes intracellular SH groups in some cells (53). We therefore examined whether another SH group oxidizing compound, diamide, would induce peeling off. Treatment of cultured cardiac myoytes or WI-38 fibroblasts with 5, 20, 50, or 200 μM diamide for 30 min did not cause peeling off. Intracellular loading of a membrane-permeating SH group reducing agent, γ-glutamylcysteine ethyl ester (a glutathione precursor) (42) (500 μM, 4 h), did not prevent NH_2Cl-induced peeling off in these cells.

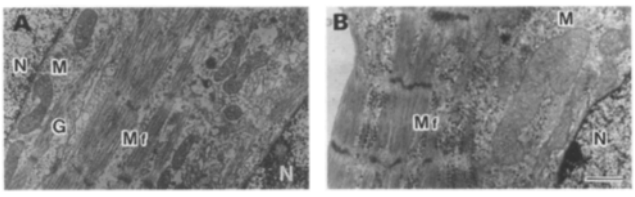

Figure 2. Transmission electron microscopy showing the effect of NH_2Cl on the ultrastructure of cultured cardiac myocytes. (*A*) Unexposed cells. (*B*) A cell exposed to 100 μM NH_2Cl for 10 min at 37°C. No ultrastructual changes in nuclei, mitochondria, and myofibrils were detected in NH_2Cl-treated cells. *N*, nucleus; *M*, mitochondria; *G*, gap junction; and Mf, myofibrils. Bar, 1 μm.

Protection of the Cells from Peeling Off by Fibronectin

We next investigated whether precoating the culture dish with Fn, laminin, and collagen types I, III, and IV, which are the main cardiac ECM proteins (1, 7, 32, 35), would protect the cardiac myocytes from peeling off. When isolated cardiac myocytes were cultured on a substratum coated with a mixture of all five of these proteins (20 μg/ml each) and exposed to NH_2Cl (100 μM), no peeling off was observed. When each of the ECM proteins was applied separately, however, only Fn had a protective effect (Fig. 3 *A*), and it was concentration dependent (Fig. 3 *B*). Fn precoating also protected fibroblasts (WI-38 cells) against NH_2Cl-induced peeling off (Fig. 4).

These observations led us to speculate that an NH_2Cl attack site might be Fn itself, and that alteration of Fn by treatment with NH_2Cl might destroy its protective effect. Culture dishes precoated with Fn that had been pretreated with a high concentration of NH_2Cl (300 μM), however, still protected cardiac myocytes against NH_2Cl-induced peeling off (Fig. 5), even though the specific viscosity of the NH_2Cl-treated Fn had decreased from 0.5 ± 0.09 (n = 4) to 0 ± 0.02 (n = 4) ($P < 0.01$). These results suggest that NH_2Cl may have changed the conformation of Fn, but this change did not affect the protein's protective activity.

Effects of NH_2Cl on the Distribution and the Contents of Focal Adhesion Proteins

Cellular adherence to the substratum is thought to be influenced by focal adhesion proteins, including p-Tyr–containing proteins and actin filaments, as well as by ECM proteins, so we next examined whether focal adhesion proteins would be affected by NH_2Cl treatment. Because Fn precoating protected the cells from peeling off, we studied the content and distribution of $\alpha_5\beta_1$ integrin, a classic Fn receptor and transmembrane component of focal adhesion. Because vinculin (a prominent intracellular component of focal adhesions) (5, 14) deficiency caused loss of cell–substratum adhesion activity in mutant mouse F9 embryonic carcinoma cells (46), we also studied the effects of NH_2Cl on the content and distribution of vinculin. Since cultured mouse myocytes were too thick for the distribution of the focal adhesion proteins or stress fibers to be observed by conventional fluorescence microscopy, and since we could only obtain antibody against human integrin, we used human fibroblast WI-38 cells, which are thinner than fetal mouse cardiac myocytes, and were able to observe integrin $\alpha_5\beta_1$, vinculin, stress fibers, and p-Tyr–containing proteins in untreated cells (Fig. 6, *A*, *C*, *E*, and *G*, respectively). Double-staining immunofluorescence microscopy of integrin $\alpha_5\beta_1$ and vinculin, of integrin $\alpha_5\beta_1$ and p-Tyr, and of vinculin and p-Tyr showed that, in general, these proteins were localized to the same part of the cell (data not shown). The organization of these proteins in WI-38 fi-

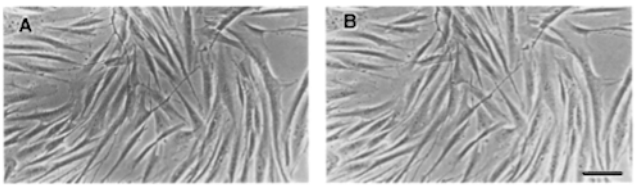

Figure 4. Protective effect of Fn precoating against NH_2Cl-induced peeling off of WI-38 fibroblasts. (*A*) An unexposed cell sheet cultured on an Fn (20 μg/ml)-coated glass coverslip. (*B*) The same cell sheet 10 min after exposure to 50 μM NH_2Cl, seen by phase-contrast microscopy. Bar, 50 μm.

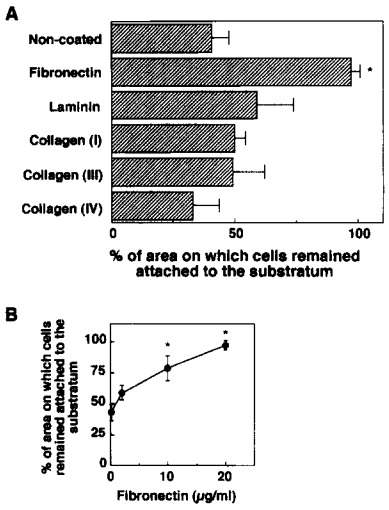

Figure 3. Protection of cardiac myocytes from NH_2Cl-induced peeling off by Fn precoating of the substratum. (*A*) Effect of precoating with several kinds of ECM proteins. Isolated mouse cardiac myocytes were cultivated on glass coverslips coated with 20 μg/ml of fibronectin, laminin, or collagen types I, III, or IV, and then exposed to 100 μM NH_2Cl for 10 min at 37°C in methionine-free MEM. The extent of peeling off is expressed as the percentage of the original cell sheet that remained attached to the substratum. Results are expressed as mean ± SEM of six experiments. * Significantly different from the results obtained without precoating ($P < 0.01$). (*B*) Concentration-dependent curve of the protective activity of Fn precoating on NH_2Cl-induced peeling off of cardiac myocytes.

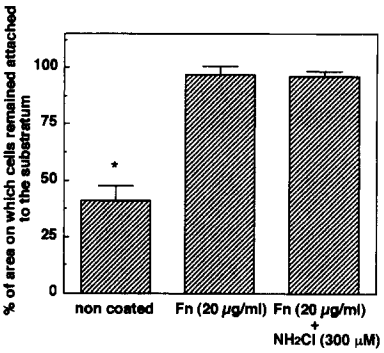

Figure 5. Protective effect of precoating with Fn that had been pretreated with NH_2Cl against NH_2Cl-induced peeling off of cardiac myocytes. 20 μg/ml Fn were pretreated in test tubes with PBS or 300 μM NH_2Cl for 30 min at 37°C and then coated on glass coverslips. Isolated mouse cardiac myocytes were cultivated on the coverslips and then exposed to 100 μM NH_2Cl for 30 min at 37°C in methionine-free MEM. The extent of peeling off is expressed as the percentage of the original cell sheet that remained attached to the substratum. Results are expressed as mean ± SEM of six experiments. * Significantly different from results obtained with the precoating of intact Fn ($P < 0.01$).

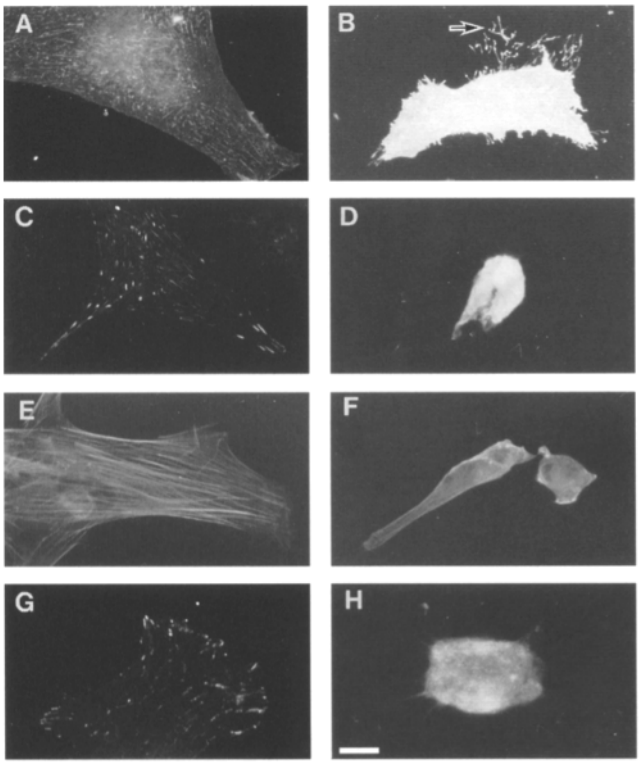

Figure 6. Fluorescence photomicrographs of WI-38 fibroblasts showing the effect of NH_2Cl on the distribution of integrin $\alpha_5\beta_1$, vinculin, stress fibers, and p-Tyr–containing proteins in Ca^{2+}-containing BSS. WI-38 cells were cultivated on glass coverslips and were untreated (*A*, *C*, *E*, and *G*) or treated (*B*, *D*, *F*, and *H*) with 50 μM NH_2Cl for 10 min at 37°C in Ca^{2+}-containing BSS. Cells were stained for integrin $\alpha_5\beta_1$ (*A* and *B*), vinculin (*C* and *D*), actin (*E* and *F*), or p-Tyr (*G* and *H*). Note that some integrins but not vinculin, actin, or p-Tyr–containing proteins left residual thin fibers of material on the substratum following peeling off. Bar, 10 μm for *A*, *C*, *E*, and *G*; 15 μm for *B*, *D*, *F*, and *H*.

broblasts as shown in Fig. 6 was quite similar to its organization in other fibroblasts (6, 19, 24).

NH_2Cl treatment of WI-38 cells altered the distribution of integrin $\alpha_5\beta_1$, vinculin, stress fibers, and p-Tyr–containing proteins (Fig. 6, *B*, *D*, *F*, and *H*, respectively). Only integrin $\alpha_5\beta_1$, however, left residual thin fibers on the substratum (Fig. 6 *B*, *arrows*), suggesting that the residual thin fibers observed by phase-contrast microscopy (Fig. 1, *D*, *F*, and *H*, *arrows*) might consist partly or wholly of integrins. Moreover, in the regions of the residual thin fibers observed by phase-contrast microscopy, no stress fibers, vinculin, or p-Tyr–containing proteins were detected by fluorescence microscopy (Fig. 6, *D*, *F*, and *H*).

To examine whether NH_2Cl-induced disruption of these proteins is accompanied by a decrease in the protein content, we compared their amount of proteins before and after NH_2Cl treatment using immunoblotting techniques. The content of integrin $\alpha_5\beta_1$, vinculin (Fig. 7 *A*, lanes *2* and *4*), and actin (43-kD protein investigated by Coomassie blue staining, data not shown) were not changed by NH_2Cl. On the other hand, p-Tyr–containing proteins were reduced (Fig. 7 *A*, lane *6*). Immunoblotting showed that the p-Tyr–containing protein $pp125^{FAK}$, however, was not changed by NH_2Cl (data not shown).

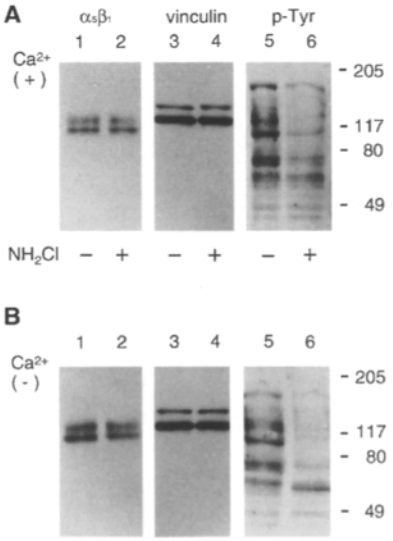

Figure 7. Effect of NH_2Cl treatment on the content of integrin $\alpha_5\beta_1$, vinculin, and p-Tyr of WI-38 fibroblasts in Ca^{2+}-containing and Ca^{2+}-free BSS. WI-38 cells were cultivated on plastic petri dishes, were untreated (lanes *1*, *3*, and *5*) or treated (lanes *2*, *4*, and *6*) with 50 μM NH_2Cl for 10 min at 37°C in Ca^{2+}-containing BSS (*A*) or in Ca^{2+}-free BSS (*B*). Cell lysates were electrophoresed on 7.5% polyacrylamide gels, transferred to nitro-cellulose, and blotted with anti-integrin $\alpha_5\beta_1$ (lanes *1* and *2*), anti-vinculin (lanes *3* and *4*), or anti–p-Tyr antibody py 20 (lanes *5* and *6*), followed by treatment with HRP-conjugated second antibody for visualization. The molecular masses of marker proteins are indicated in kilodaltons on the right.

Role of p-Tyr Dephosphorylation in Induction of Peeling Off

To examine the role of p-Tyr dephosphorylation on NH_2Cl-induced peeling off, we examined the effects of inhibitors of both tyrosine kinase and protein–tyrosine phosphatase (PTPase) on the action of NH_2Cl. WI-38 fibroblasts were treated with a specific tyrosine kinase inhibitor, genistein (2) (100 μM), for 6 h in FBS-MEM at 37°C. Genistein treatment alone did not induce peeling off (Fig. 8 *D*), although most of the p-Tyr–containing proteins disappeared with this treatment (Fig. 8 *B*). On the other hand, when the cells were preincubated with a PTPase inhibitor Na_3VO_4 (1 mM) for 1 h at 37°C, no peeling off was induced by subsequent exposure to 50 μM NH_2Cl in the absence of Na_3VO_4 (Fig. 9 *B*). Na_3VO_4 pretreatment actually prevented the NH_2Cl-induced loss of p-Tyr (Fig. 9 *D*). These observations suggest that dephosphorylation of p-Tyr is necessary, but not sufficient for induction of peeling off.

To determine whether NH_2Cl affects PTPase activity, we measured the PTPase activity of in the lysates of both 50 μM NH_2Cl-treated and untreated WI-38 cells using ^{32}P-Raytide or pNPP as the substrate. The PTPase activities of the lysates were similar: (^{32}P-Raytide; nontreated: 1856 ± 86 cpm; treated: 1905 ± 77 cpm, $n = 3$)) (pNPP, OD_{410nm}, nontreated: 1.03 ± 0.05; treated: 1.02 ± 0.06, $n = 3$).

Role of Polymerized Actin in Induction of Peeling Off

Because some reports suggest that cell detachment is ac-

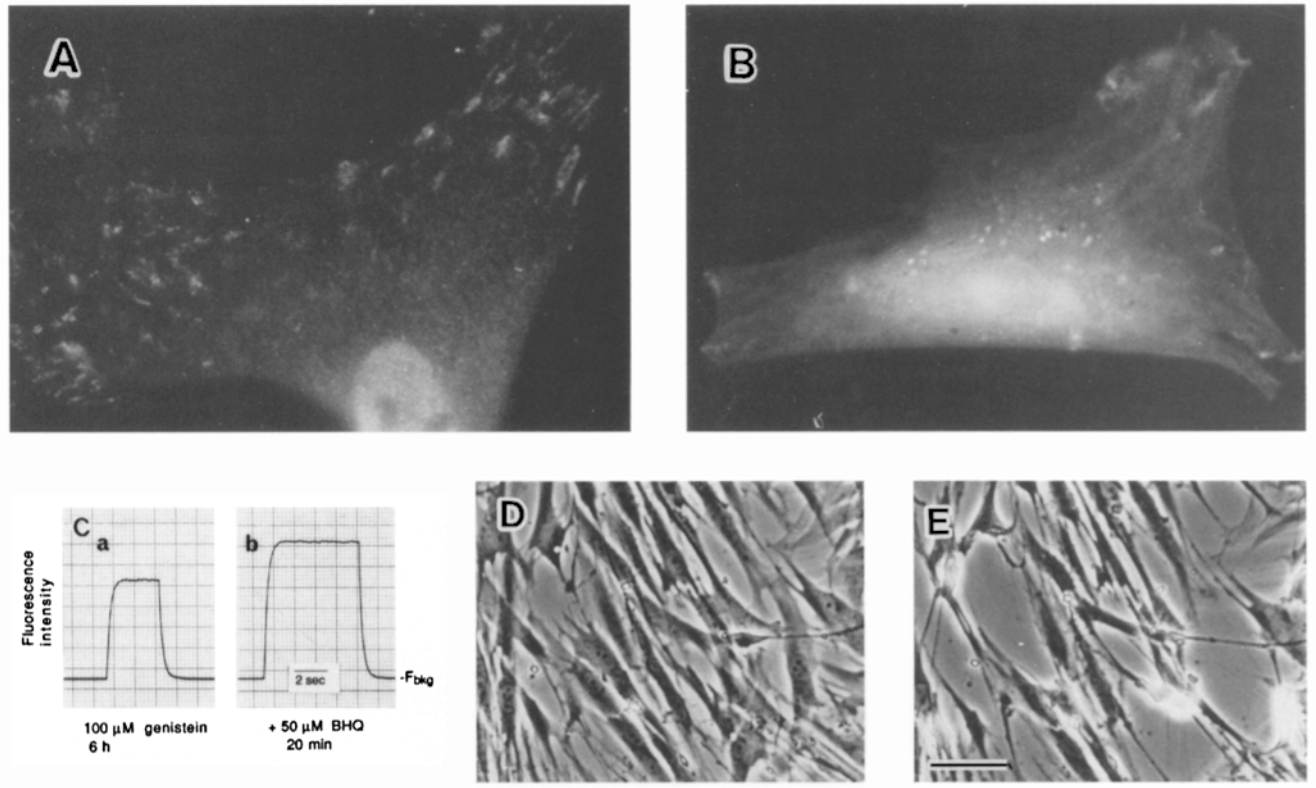

Figure 8. Effect of the tyrosine kinase inhibitor genistein and the microsomal Ca^{2+}-ATPase inhibitor BHQ on the distribution of p-Tyr–containing proteins, $[Ca^{2+}]_i$ and cell–substratum adhesion of WI-38 fibroblasts. WI-38 cells cultured on glass coverslips were treated with 100 μM genistein for 6 h in FBS-MEM. After washing with MEM, they were exposed to 50 μM BHQ for 20 min. (*A* and *B*): Fluorescence photomicrographs showing the distribution of p-Tyr–containing proteins of genistein-untreated (*A*) and treated (*B*) WI-38 cells. After the treatment, cells were stained for p-Tyr as described in the legend of Fig. 6. (*C*): The $[Ca^{2+}]_i$ of genistein-treated (*a*) (138 ± 21 nM [n = 4]) and subsequent BHQ-treated WI-38 cells (*b*) (220 ± 18 nM [n = 4]). F_{bkg}, the background signal. (*D* and *E*) Phase-contrast microscopy of genistein-treated (*D*) and subsequent BHQ-treated WI-38 cells (*E*). Bars, 10 μm for *A*; 15 μm for *B*; 50 μm for *D* and *E*. Similar results were obtained in four to six other experiments.

companied by depolymerization of actin filaments (4, 54), we examined the effect of NH_2Cl on the viscosity of F-actin in vitro. NH_2Cl treatment did not significantly change the specific viscosity of purified F-actin (0.86 ± 0.03, n = 6) relative to that of nontreated protein (0.92 ± 0.05, n = 6), although cytochalasin D (a potent actin-depolymerizing agent) did (0.10 ± 0.01, n = 6) ($P < 0.01$). In addition, incubation of WI-38 cells or cardiac myocytes with cytochalasin D (0.5 μM, 30 min at 37°C; this condition can induce disruption of stress fibers in WI-38 cells) did not induce peeling off (data not shown). These observations suggest that NH_2Cl-induced disruption of stress fibers was not due to the depolymerization of F-actin and that such disruption alone does not induce peeling off. Furthermore, pretreatment of WI-38 cells with cytochalasin D but not with colcemid (a potent tubulin-depolymerizing agent) (5 μM, 10 μM, 30 min at 37°C), protected the cells from NH_2Cl-induced peeling off, suggesting that the presence of polymerized actin is necessary for the peeling off process.

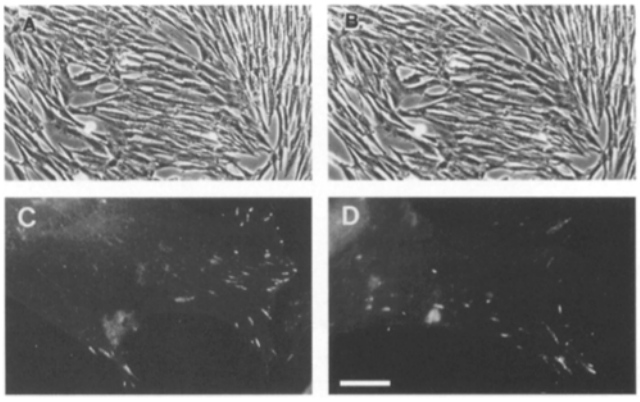

Figure 9. Effect of preincubation with the p-Tyr phosphatase inhibitor Na_3VO_4 on NH_2Cl-induced peeling off of WI-38 fibroblasts. WI-38 cells were cultivated on glass coverslips and preincubated with 1 mM Na_3VO_4 for 1 h at 37°C (*A*). After washing with methionine-free MEM (containing no Na_3VO_4), they were exposed to 50 mM NH_2Cl for 10 min (*B*), and viewed by phase-contrast microscopy. Na_3VO_4-preincubated untreated (*C*) and NH_2Cl-treated cells (*D*) were stained for p-Tyr as described in the legend of Fig. 6 and viewed by fluorescent microscopy. Bars, 100 μm for *A* and *B*; 10 μm for *C* and *D*.

Role of Ca^{2+} for Induction of Peeling Off

The observations that NH_2Cl-induced peeling off was accompanied by cellular shrinkage, that it did not occur under 15°C, and that polymerized actin was necessary

suggest that actin-dependent cellular motility might play a role in the induction of peeling off. Because cellular motility requires extracellular Ca^{2+}, we studied the effect of Ca^{2+}-free medium on the action of NH_2Cl. When WI-38 cells were exposed to NH_2Cl (50 μM) in Ca^{2+}-free BSS containing EGTA, no peeling off was observed (Fig. 10 *B*). Subsequent incubation of the cells in Ca^{2+}-containing BSS (containing no NH_2Cl), however, caused severe peeling off within 30 s (Fig. 10 *C*). Treatment of the cells with NH_2Cl (50 μM) in Mg^{2+}-free but Ca^{2+}-containing BSS resulted in peeling off (data not shown). Similar results were observed in cardiac myocytes (data not shown). These observations indicate that extracellular Ca^{2+}, too, is necessary for the peeling off process.

To study in more detail the relationship between Ca^{2+} and peeling off, we measured the $[Ca^{2+}]_i$ of cultured fetal mouse cardiac myocytes and WI-38 cells. In control experiments, we observed that $[Ca^{2+}]_i$ did not change in either WI-38 cells or fetal mouse cardiac myocytes during a 60-min superfusion with Ca^{2+}-containing BSS, indicating that there was no extinction of fluorescent light in this condition (41). As shown in Fig. 11 *A*, the diastolic and systolic $[Ca^{2+}]_i$ of the myocytes in Ca^{2+}-containing BSS were 85 ± 16 (n = 10) and 380 ± 62 nM (n = 10), respectively. On treatment with NH_2Cl, the diastolic value increased to 390 ± 50 nM (n = 8), although this was almost the same as the systolic $[Ca^{2+}]_i$ level of normally beating cells (Fig. 11 *A b*). Treatment of WI-38 cells with NH_2Cl in Ca^{2+}-containing BSS increased the $[Ca^{2+}]_i$ from 136 ± 20 (n = 8) to 244 ± 41 nM (n = 8) (Fig. 11 *B*). When WI-38 cells were incubated in Ca^{2+}-free BSS, $[Ca^{2+}]_i$ decreased from 136 ± 20 (n = 8) to 74 ± 16 nM (n = 4) (Fig. 11 *C b*); the addition of NH_2Cl (50 μM) did not change the $[Ca^{2+}]_i$ (Fig. 11 *C c*). Subsequent incubation of the cells in Ca^{2+}-containing BSS (containing no NH_2Cl), however, resulted in an apparent increase in $[Ca^{2+}]_i$ (241 ± 45 nM [n = 4]), (Fig. 11 *C d*), which was higher than the control level (136 ± 20 nM) (Fig. 11 *C a*) . In all the above experiments, peeling off was concomitant with the increase in $[Ca^{2+}]_i$.

Since Na_3VO_4 is not only a PTPase inhibitor, but also an inhibitor of various kinds of ATPase, including Na^+-K^+ ATPase and Ca^{2+}-ATPase (33), we examined the effect of Na_3VO_4 on the $[Ca^{2+}]_i$ of WI-38 fibroblasts, to make sure that the protective effect of Na_3VO_4 on NH_2Cl-induced peeling off was not due to decrease in $[Ca^{2+}]_i$. Treatment of WI-38 fibroblasts with 1 mM Na_3VO_4 for 1 h caused a slight (but not significant) increase in $[Ca^{2+}]_i$ of the cells (control, 134 ± 15 nM [n = 12]; Na_3VO_4 treated, 151 ± 13 nM [n = 8]), though this treatment prevented both NH_2Cl-induced disappearance of p-Tyr–containing proteins (Fig. 9 *D*) and peeling off (Fig. 9 *B*).

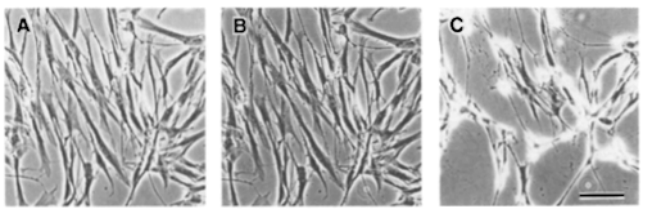

Figure 10. Effect of extracellular Ca^{2+} on NH_2Cl-induced peeling off of WI-38 fibroblasts. WI-38 cells cultivated on glass coverslips were preincubated in Ca^{2+}-free BSS containing EGTA for 5 min (*A*) and then treated with 50 μM NH_2Cl for 10 min at 37°C in the same buffer (Ca^{2+}-free) (*B*). The same cell sheet 30 s after the incubation medium was changed to Ca^{2+}-containing BSS (containing no NH_2Cl) (*C*), seen by phase-contrast microscopy. Bar, 50 μm.

We also studied the protein content and distribution of focal adhesion proteins in WI-38 cells treated with NH_2Cl in Ca^{2+}-free media. The distribution of integrin $\alpha_5\beta_1$, vinculin, and stress fibers did not change (Fig. 12, *B*, *D* and *F*, respectively). On the other hand, most of p-Tyr–containing proteins disappeared even though the cells did not peel off the substratum (Fig. 12 *H*).

The protein content of integrin $\alpha_5\beta_1$, vinculin (Fig. 7 *B*, lanes *2* and *4*) and actin (43-kD protein investigated by Coomassie blue staining, data not shown) were not changed by treatment with NH_2Cl in Ca^{2+}-free BSS containing EGTA. The amount of p-Tyr, however, was apparently reduced (Fig. 7 *B*, lane *6*).

Effects of Ca^{2+} Entry Inhibitors on NH_2Cl-induced Peeling Off

To study the mechanism of the NH_2Cl-induced increase in $[Ca^{2+}]_i$, we examined the effect of various kinds of Ca^{2+} entry inhibitors on the action of NH_2Cl. One possible mechanism of increasing $[Ca^{2+}]_i$ is Ca^{2+} entry from the extracellular medium via Ca^{2+} channels, and Na^+–Ca^{2+} exchange systems. Na^+–H^+ exchange is also involved in Ca^{2+} entry from the extracellular medium in cooperation with Na^+–Ca^{2+} exchange systems. Another possibility is that Ca^{2+} is released from intracellular Ca^{2+} storage sites, such as microsomes. Preincubation of WI-38 fibroblasts and cardiac myocytes with Ca^{2+} channel blockers (verapamil or nifedipine) did not prevent NH_2Cl-induced peeling off (Table I). Preincubation with the Na^+–Ca^{2+} exchange inhibitor bepridil (13), or with the Na^+–H^+ exchange inhibitor amiloride (59), also did not prevent NH_2Cl-induced peeling off (Table I). When WI-38 fibroblasts were treated with the microsomal Ca^{2+}-ATPase inhibitor BHQ (26) in Ca^{2+}-containing BSS, however, the cytosolic Ca^{2+} concentration increased from 136 ± 20 (n = 12) to 258 ± 24 (n = 4) (Fig. 11 *D b*); after this incubation of these cells in Ca^{2+}-free medium containing BHQ lowered $[Ca^{2+}]_i$ to 45 ± 11 nM (n = 4) (Fig. 11 *D c*). Subsequent exposure of the cells to NH_2Cl in Ca^{2+}-BSS containing BHQ did not induce peeling off (Table I), and $[Ca^{2+}]_i$ increased slightly but not significantly ($P > 0.2$) (Fig. 11 *D d*). The same results were obtained in cultured cardiac myocytes (data not shown). These observations suggest that microsomal Ca^{2+} mobilization is involved in the NH_2Cl-induced increase in $[Ca^{2+}]_i$ and peeling off.

The next problem is how microsomal Ca^{2+} was moved from the microsomes to the cytosol by NH_2Cl. Although transient increase in inositol 1, 4, 5-triphosphate ($InsP_3$) levels can induce Ca^{2+} release from intracellular Ca^{2+} stores (3), NH_2Cl did not increase intracellular $InsP_3$ levels in WI-38 cells or cardiac myocytes (data not shown). Another possibility is that NH_2Cl directly inhibits the Ca^{2+}-pump of the intracellular Ca^{2+}-storage site membranes. We examined the effect of NH_2Cl in vitro on the active Ca^{2+} transport using skeletal muscle sarcoplasmic reticu-

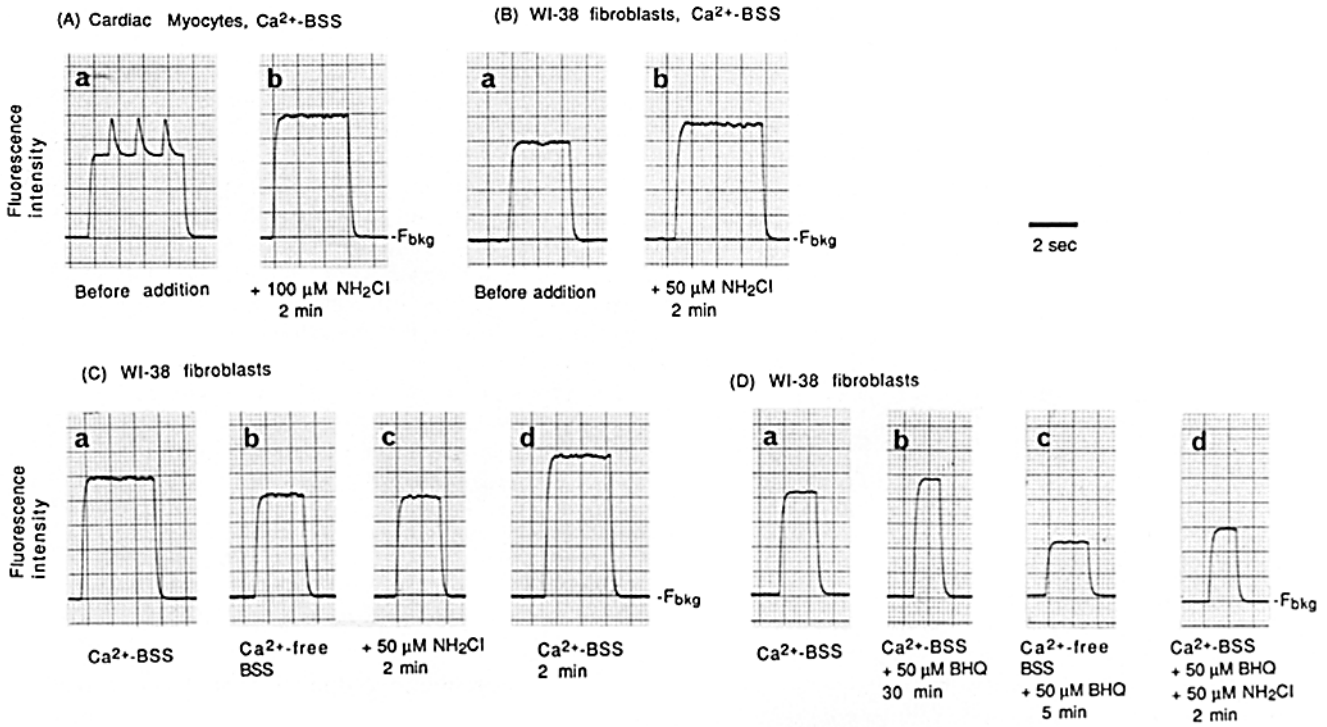

Figure 11. Effect of NH_2Cl on $[Ca^{2+}]_i$ of cardiac myocytes and WI-38 fibroblasts in Ca^{2+}-containing BSS and Ca^{2+}-free BSS containing EGTA. (*A*) NH_2Cl-induced increase in $[Ca^{2+}]_i$ of cardiac myocytes in Ca^{2+}-containing BSS. Cardiac myocytes showed spontaneous beating before the addition of NH_2Cl (*a*). Treatment with 100 μM NH_2Cl caused cessation of spontaneous beating and an increase in diastolic $[Ca^{2+}]_i$ within 2 min, although the $[Ca^{2+}]_i$ level was almost the same as it was in normal systole (*b*). The cells showed peeling off at 2 min treatment (phase-contrast microscopy). (*B*) NH_2Cl-induced increase in $[Ca^{2+}]_i$ of WI-38 fibroblasts in Ca^{2+}-containing BSS. Treatment with 50 μM NH_2Cl caused both an increase in $[Ca^{2+}]_i$ (*b*) and peeling off within 2 min. (*C*) Effect of Ca^{2+}-free medium on the NH_2Cl-induced increase in $[Ca^{2+}]_i$ of WI-38 fibroblasts. Cells were incubated in Ca^{2+}-containing BSS (*a*). Changing the medium to Ca^{2+}-free BSS containing EGTA caused a decrease in $[Ca^{2+}]_i$ (*b*). Treatment with 50 μM NH_2Cl for 2 min caused neither a change in $[Ca^{2+}]_i$ (*c*) nor peeling off. Subsequent incubation of the cells in Ca^{2+}-containing BSS caused an increase in $[Ca^{2+}]_i$ within 30 s (*d*), which was higher than the control level (*a*). Peeling off was concomitantly observed. (*D*) Effect of the microsomal Ca^{2+}-ATPase inhibitor, BHQ, in the presence or absence of NH_2Cl on $[Ca^{2+}]_i$ of WI-38 fibroblasts. WI-38 fibroblasts in Ca^{2+}-containing BSS (*a*) were incubated in 50 μM BHQ for 30 min (*b*) and subsequently incubated in Ca^{2+}-free BSS containing EGTA and BHQ for 5 min (*c*). This caused depletion of both microsomal and cytosolic Ca^{2+}. Then the cells were treated with 50 μM NH_2Cl in Ca^{2+}-free BSS containing BHQ. F_{bkg}, the background signal. Similar results were obtained in three to nine other experiments.

lum vesicles, and found that NH_2Cl inhibited ATP-dependent Ca^{2+} uptake rapidly (<10 s) (Fig. 13). Half maximal inhibition occurred at about 20 μM NH_2Cl, and >90% of the Ca^{2+} uptake activity was inhibited by 50 μM NH_2Cl (Fig. 13). NH_2Cl did not cause leakage of $^{45}Ca^{2+}$ from the vesicles (Fig. 13), however, indicating that NH_2Cl inhibits Ca^{2+}-pump activity by interacting directly with the Ca^{2+}-pump molecule, not by making the membrane leaky. These data strongly suggest that NH_2Cl-induced Ca^{2+}-mobilization is caused by the inhibition of the Ca^{2+}-pump in the intracellular Ca^{2+} storage sites.

Synergistic Role of p-Tyr Dephosphorylation and Increase in $[Ca^{2+}]i$ on Induction of Peeling Off

To confirm the synergism between p-Tyr dephosphorylation and increased $[Ca^{2+}]_i$ on NH_2Cl-induced peeling off, we examined the combined effect of two agents: one that induces p-Tyr dephosphorylation and one that increases $[Ca^{2+}]_i$. Treatment of WI-38 fibroblasts with 100 μM genistein (a tyrosine kinase inhibitor) (2) for 6 h in FBS-MEM at 37°C caused p-Tyr dephosphorylation (Fig. 8 *B*), and subsequent treatment of the cells with 50 μM BHQ (a microsomal Ca^{2+}-ATPase inhibitor) (26) for 20 min in MEM at 37°C caused an increase in $[Ca^{2+}]_i$ (Fig. 8 *C b*) concomitant with the induction of peeling off (Fig. 8 *E*). This observation that the effect of genistein + BHQ mimicked that of NH_2Cl suggests that NH_2Cl-induced peeling off is explained by synergism between p-Tyr dephosphorylation and an increase in $[Ca^{2+}]_i$. Genistein treatment did not induce disorganization of integrin $\alpha_5\beta_1$ or vinculin (data not shown).

Protective Effect of Fn-Precoating against NH_2Cl-induced Dephosphorylation of p-Tyr

We next measured the p-Tyr content of WI-38 cells cultured on Fn-coated dishes. As shown in Fig. 14 (lane *2*), treatment with NH_2Cl in Ca^{2+}-containing BSS caused no change in the cellular p-Tyr content. Prevention of NH_2Cl-induced p-Tyr dephosphorylation may be the basis for the protective effect of Fn coating.

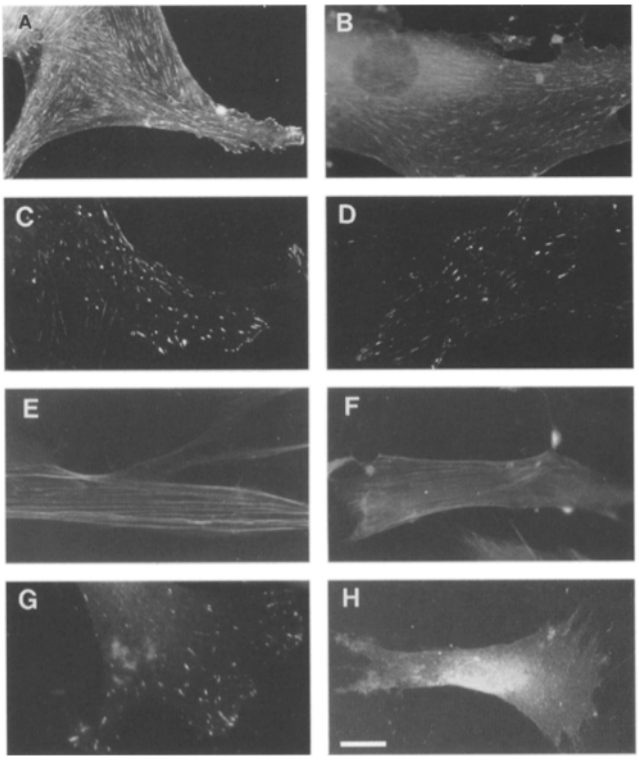

Figure 12. Fluorescence photomicrographs of WI-38 fibroblasts showing the effects of NH_2Cl on the distribution of integrin $\alpha_5\beta_1$, vinculin, stress fibers, and p-Tyr–containing proteins in Ca^{2+}-free BSS containing EGTA. WI-38 cells cultivated on glass coverslips were preincubated in Ca^{2+}-free BSS containing EGTA for 5 min and then they were untreated (*A*, *C*, *E*, and *G*) or treated (*B*, *D*, *F*, and *H*) with 50 μM NH_2Cl in the same buffer (Ca^{2+}-free BSS containing EGTA) for 10 min at 37°C. Cells were stained for integrin $\alpha_5\beta_1$ (*A* and *B*), vinculin (*C* and *D*), actin (*E* and *F*), or p-Tyr (*G* and *H*). Note that the distribution of integrin $\alpha_5\beta_1$, vinculin, and stress fibers was not changed by NH_2Cl treatment when the cells were treated in Ca^{2+}-free BSS, but the distribution of p-Tyr was reduced. Bars, 10 μm for *A*, *B*, *C*, *D*, and *G*; 15 μm for *E* and *F*; 18 μm for *H*.

Stimulated Neutrophils Induced Peeling Off of Cardiac Myocytes

We next examined whether stimulated human neutrophils induce the peeling off of cultured fetal mouse cardiac myocytes. Since stimulated neutrophils produce several kinds of cytotoxic mediators such as ·OH, NH_2Cl and elastase, cardiac myocytes were treated with neutrophils in the presence of inhibitor(s) against each of these mediators separately to see each of their cytotoxic effects. On treatment with stimulated neutrophils in the absence of any inhibitors, 10–18% of the cardiac myocytes showed morphological degeneration characterized by hypercontraction concomitant with balloon formation (Fig. 15 *A*). These morphologically degenerated cells were stained with the vital dye erythrosine B. An elastase inhibitor, α_1-antitrypsin, did not prevent the neutrophil-induced morphological changes (data not shown). On the other hand, in the presence of α_1-antitrypsin, DMTU (an ·OH scavenger) (12), and deferoxamine (an iron chelator [37] and an inhibitor of ·OH production) stimulated neutrophils induced peeling off of 13–15% of the cardiac myocytes (Fig. 15 *B*) in the absence of balloon formation and hypercontraction. These peeled-off cells excluded erythrosine B, and the morphological change was reversible, that is, when the incubation medium was changed to FBS-MEM and the cells cultivated for 24 h, almost all myocytes readhered to and spread on the substratum and resumed spontaneous beating. Coexistence of α_1-antitrypsin, DMTU, deferoxamine, and methionine (an NH_2Cl scavenger) prevented both types of neutrophil-induced morphological changes in cardiac myocytes, even though some neutrophils adhered to them (Fig. 15 *C*). Adherence of neutrophils to cardiac myocytes was essential, but not sufficient to induce either hypercontraction or peeling off of the myocytes. These observations suggest that neutrophil-derived NH_2Cl also caused peeling off of the cultured cardiac myocytes.

Table I. Effects of Various Ca^{2+} Entry Inhibitors on NH_2Cl-induced Peeling Off

	NH_2Cl-induced peeling off	
Ca^{2+} entry inhibitors	WI-38 fibroblasts	Cardiac myocytes
Ca^{2+} channel blockers		
Verapamil (1, 10, 100 μM, 10 min)	Not prevented	Not prevented
Nifedipine (1, 10, 100 μM, 10 min)	Not prevented	Not prevented
Na^+-Ca^{2+} exchange inhibitor		
Bepridil (10, 30 μM, 10 min)	Not prevented	Not prevented
Na^+-H^+ exchange inhibitor		
Amiloride (100 μM, 30 min)	Not prevented	Not prevented
Microsomal Ca^{2+}-ATPase inhibitor		
BHQ (50 μM, 30 min for WI-38)		
(20 μM, 15 min for myocytes)	Prevented	Prevented

WI-38 fibroblasts and cardiac myocytes were preincubated with Ca^{2+} channel blockers, Na^+–Ca^{2+} exchange inhibitor, or Na^+–H^+ exchange inhibitor, and then exposed to 50 μM NH_2Cl for WI-38 fibroblasts or 100 μM NH_2Cl for cardiac myocytes. To examine the effect of the microsomal Ca^{2+}-ATPase inhibitor BHQ, these cells were treated with BHQ in Ca^{2+}-BSS for 30 (WI-38 fibroblasts) or 15 min (cardiac myocytes), and then incubated in Ca^{2+}-free BSS containing EGTA and BHQ for 5 min. The cells were then exposed to NH_2Cl in Ca^{2+}-BSS containing BHQ.

The next interesting point is that peeling off was not detected in the myocytes 2.5–3 h after exposure to stimulated neutrophils in the absence of inhibitors (Fig. 15 *A*), although NH_2Cl must have still existed in that condition. To see the additive effect of ·OH and NH_2Cl, we examined the additive effect of low concentrations of H_2O_2 and NH_2Cl on the morphology of cultured cardiac myocytes. Neither 20 μM H_2O_2 nor 25 μM NH_2Cl alone caused hypercontraction or peeling off of the myocytes during the 2-h observation period. On the other hand, application of both 20 μM H_2O_2 and 25 μM NH_2Cl caused peeling off of 50–60% of the myocytes in 30 min after application, although this treatment did not induce hypercontraction. After incubation for another 30 min, however, 30–40% of the myocytes showed hypercontraction concomitant with balloon formation, and 20–30% of remaining cells showed peeling off. In 1.5–2.0 h after the addition of both 20 μM H_2O_2 and 25 μM NH_2Cl, 60–70% of the cells showed hypercontraction, and no peeled-off cells were detected (data not shown). These observations indicate that (*a*) H_2O_2 enhanced the effect of NH_2Cl to induce peeling off, (*b*) NH_2Cl enhanced the effect of H_2O_2 to induce hypercontraction, and (*c*) the peeled-off cells (but not other in-

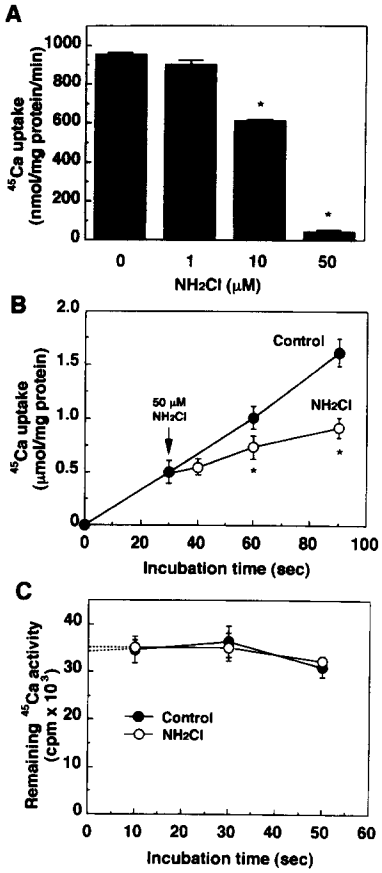

Figure 13. Effect of NH_2Cl on the ATP-dependent Ca^{2+} pump and Ca^{2+} release from sarcoplasmic reticulum vesicles. (*A*) ATP-dependent $^{45}Ca^{2+}$ uptake was measured for 3 min in the presence of various concentrations of NH_2Cl as described in Materials and Methods. (*B*) The time course of ATP-dependent $^{45}Ca^{2+}$ uptake was measured. At the time shown by the arrow, 50 μM NH_2Cl was added. (*C*) $^{45}Ca^{2+}$-loaded vesicles were diluted into the buffer containing 10 mM $MgCl_2$ and 10 μM ruthenium red in the absence or presence of 50 μM NH_2Cl, and remaining $^{45}Ca^{2+}$ radioactivity was measured as described in Materials and Methods. * Significantly different from the results obtained with control ($P < 0.01$).

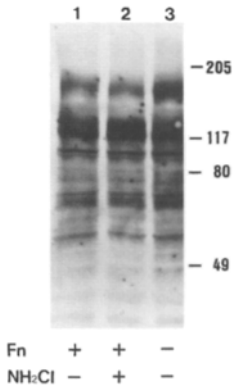

Figure 14. Effect of Fn precoating on the p-Tyr content of WI-38 fibroblasts. WI-38 cells were cultivated on the plastic petri dishes coated with (lanes *1* and *2*) or without (lane *3*) 20 μg/ml Fn and then were untreated (lanes *1* and *3*) or treated (lane *2*) with 50 μM NH_2Cl for 10 min at 37°C. Cell lysates were electrophoresed on 7.5% polyacrylamide gel, transferred to nitrocellulose, and blotted with the anti-p-Tyr antibody py 20 followed by HRP-conjugated second antibody for visualization. The molecular masses of marker proteins are indicated in kilodaltons on the right hand side.

tact cells) progressed to hypercontraction. This suggests that in the neutrophil treatment experiment, the neutrophil-derived, NH_2Cl-induced peeling off of cultured cardiac myocytes may contribute to the formation of neutrophil-induced hypercontraction.

Discussion

Interaction with extracellular adhesion molecules plays a critical role in regulating cellular morphology, proliferation, migration, and differentiation. Alterations in myocyte basement membrane attachments and in the distribution of ECM components were observed in heart failures, such as experimental tachycardia (63) and pressure overload–induced hypertrophy (7), respectively, indicating the importance of cell–substratum adhesion for normal heart function. Ample evidence suggests that activated neutrophils accumulate in ischemic regions of the heart during the early h of reperfusion (9) and might mediate tissue injuries (40, 45). When an MPO–H_2O_2-Cl^- system (HOCl- and NH_2Cl-generating system) was infused into rat kidneys, it induced tissue injury and morphological abnormalities (22, 23). These findings suggest the possibility that neutrophil-derived oxidants inhibit cell–substratum adhesion. Previous studies showing that NH_2Cl induced detachment of cultured cardiac myocytes (41) and endothelial cells (54) from the substratum support this hypothesis. In the present study, we showed that stimulated neutrophils, in fact, induced peeling off (cell detachment and cell shrinkage) of cultured cardiac myocytes when the myocytes were treated with neutrophils in the presence of both inhibitors of ·OH and elastase (Fig. 15 *B*). We also found

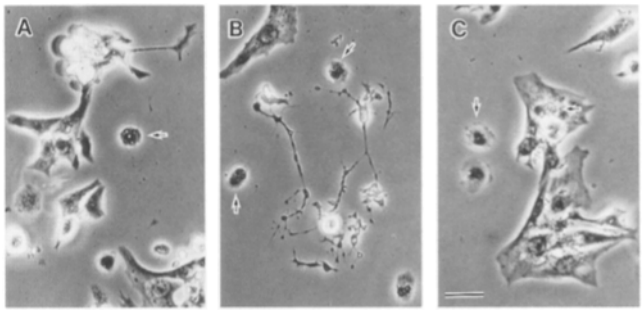

Figure 15. Effect of stimulated neutrophils on the morphology of cultured fetal mouse cardiac myocytes. Cultured fetal mouse cardiac myocytes (10^5 cells per dish) were pretreated with or without various inhibitors, that is, the iron chelator deferoxamine (10 mM, for 6 h), the ·OH scavenger DMTU (10 mM, for 30 min), the elastase inhibitor α_1-antitrypsin (50 μg/ml, for 30 min) and/or the NH_2Cl scavenger methionine (10 mM, for 30 min). After treatment with PAF (10^{-6} M) for 30 min in HBSS, the myocytes were coincubated with human neutrophils (2×10^6 cells per dish) for 30 min to be adhered to each other, and then the neutrophils were stimulated by exposure to both PMA (100 ng/ml) and TNF-α (10 ng/ml). The morphological changes of the myocytes were assessed by phase-contrast microscopy at 2.5 to 3 h after exposure to neutrophils. (*A*) The cell sheets pretreated with no inhibitors. Hypercontraction concomitant with balloon formation was evident. (*B*) The cell sheets pretreated with deferoxamine, DMTU and α_1-antitrypsin. Peeling off was observed. (*C*) The cell sheets pretreated with deferoxamine, DMTU, α_1-antitrypsin and methionine. No morphological changes were observed. Arrows indicate neutrophils. Bar, 20 μm.

in this study that NH_2Cl-induced peeling off was not specific to cardiac myocytes or endothelial cells, but was also observed in other kinds of cells, including fibroblasts and epithelial cells. These morphological changes were accompanied by the disorganization of integrin $\alpha_5\beta_1$, vinculin, stress fibers, and p-Tyr–containing proteins, and by an increase in $[Ca^{2+}]_i$. We suggest a synergistic relationship between p-Tyr dephosphorylation and increased $[Ca^{2+}]_i$ in NH_2Cl-induced peeling off.

The observation that residual bits of integrin $\alpha_5\beta_1$, but not of vinculins or stress fibers, remained on the substratum after NH_2Cl-induced peeling off (Fig. 6) suggests that NH_2Cl does not inhibit the interaction between integrin $\alpha_5\beta_1$ and Fn. NH_2Cl penetrates cells rapidly (53), and we observed that taurine-NHCl, a potent oxidizing but non–membrane-permeating chloramine (55), did not cause peeling off, indicating that penetration of the oxidant is required to induce peeling off, i.e., that the attack site of NH_2Cl may be intracellular.

Role of Dephosphorylation of p-Tyr for Induction of Peeling Off

A great deal of data suggests that tyrosine phosphorylation of focal contact proteins plays a critical role in regulating adhesion, motility, and shape in various kinds of cells (24). It has been reported that such phosphorylation is an early cellular response to plating onto Fn-coated dishes (18). In this study, we showed that a decrease in the protein p-Tyr content of the cells accompanied NH_2Cl-induced cellular detachment from the substratum (Figs. 6 and 7). This finding agrees with the previous report that conditions leading to decreased cell–substratum adhesion (by treatment with trypsin or Ca^{2+}- and Mg^{2+}-free PBS) result in a loss of focal adhesion protein p-Tyr (34).

We also observed in this study that inhibition of p-Tyr phosphatase activity by Na_3VO_4 protected WI-38 cells from NH_2Cl-induced peeling off (Fig. 9), suggesting that dephosphorylation of p-Tyr is essential for peeling off. It is not clear how NH_2Cl induced p-Tyr dephosphorylation in the present study. Dephosphorylation of p-Tyr can be explained by either an increase in p-Tyr phosphatase (PTPase) activity or a decrease in protein tyrosine kinase activity, or both. It is reported that PTPases are activated when cell–substratum adhesion is disrupted by trypsin in chicken embryo fibroblasts (34). In the present study, however, no increase in PTPase activity was observed in cell lysates following treatment of the cells with NH_2Cl. There are some differences between the effects of NH_2Cl and trypsin; trypsin inhibits cell–cell adhesion as well as cell–substratum adhesion, whereas NH_2Cl inhibits cell–substratum adhesion and induces cell shrinkage. Therefore, the mechanisms by which NH_2Cl and trypsin cause cell to detach from the substratum may be different. We still cannot exclude the possibility, however, that NH_2Cl increased some PTPase activity in the cells. In human cells, >20 different PTPases are present, but little is known about their substrates. Because the PTPase activities measured in this study are means of the activities of all these PTPases, it is difficult to determine the activity of a specific PTPase, even if NH_2Cl increased the activity of only some PTPases. The difference of the substrate used in this experiment (^{32}P-labeled Raytide or pNPP) from the in vivo substrate might also account for these results.

It is also not clear whether tyrosine kinase activity was changed by NH_2Cl in the present study. The observation that p-Tyr dephosphorylation by a tyrosine kinase inhibitor (genisteine) plus increase in $[Ca^{2+}]_i$ by BHQ induced peeling off (Fig. 8) suggests that p-Tyr dephosphorylation via inhibition of tyrosine kinase activity as well as activation of PTPase may be related to the induction of peeling off. Another tyrosine kinase inhibitor, herbimycin A, inhibits $pp60^{v\text{-}src}$, a tyrosine kinase in Rous sarcoma virus (44), by binding reactive SH groups and blocking an active site (57). Also, NH_2Cl lowers acid-soluble SH levels in rat colonic mucosa (53), indicating that oxidation of intracellular thiols occurred in the treated cells. Therefore, it is possible that SH-group oxidizing compounds inhibited tyrosine kinase activity just as herbimycin A does. In the present study, however, treatment of cultured cardiac myocytes or WI-38 fibroblasts with a membrane-permeating SH-group oxidizing compound (diamide) did not cause peeling off. Intracellular loading of an SH-group reducing agent (γ-glutamylcysteine ethyl ester, a glutathione precursor) (42) also did not affect NH_2Cl-induced peeling off. So, it is still unclear whether SH-group oxidation took part in NH_2Cl-induced peeling off in this study.

Role of Ca^{2+} for Induction of Peeling Off

In the present study, we found that NH_2Cl treatment of WI-38 cells or cardiac myocytes in Ca^{2+}-containing BSS induced an increase in $[Ca^{2+}]_i$ (Fig. 11) along with peeling off, although in the case of cardiac myocytes, the increased $[Ca^{2+}]_i$ did not exceed the systolic level of spontaneously beating cells (Fig. 11 *A b*). This observation does not contradict the observation that neither hypercontraction nor contraction bands of myofibrils were detected in the peeled-off myocytes (Fig. 2 *B*). Our previous study showed that when the $[Ca^{2+}]_i$ of cultured myocytes exceeded 1 μM, hypercontraction did appear (41).

In Ca^{2+}-free BSS containing EGTA, in contrast to Ca^{2+}-containing BSS, NH_2Cl induced neither peeling off nor an increase in $[Ca^{2+}]$i, but subsequent incubation of the cells in Ca^{2+}-containing BSS without NH_2Cl caused both peeling off and a concomitant increase in $[Ca^{2+}]_i$ (Figs. 10 and 11 *C*). These observations suggest that an increase in $[Ca^{2+}]_i$ plays a pivotal role on the action of NH_2Cl. This speculation agrees with the previous report that NH_2Cl-induced short-term Cl^- secretion in cultured T84 monolayers was accompanied by an increase in intracellular Ca^{2+}, and the absence of Ca^{2+} from the medium inhibited the secretion response (52). The observation that peeling off occurred in Ca^{2+}-containing BSS even in the absence of NH_2Cl if the cells had previously been treated with NH_2Cl (Fig. 10), also suggests that the NH_2Cl reaction occurs intracellularly.

In Ca^{2+}-free BSS containing EGTA, although no disorganization and no decrease of integrin, vinculin, or stress fibers followed NH_2Cl treatment, the decrease in the p-Tyr content of the cells was the same as in Ca^{2+}-containing BSS. One possible explanation for this is that the Ca^{2+}-free condition itself caused the dephosphorylation of p-Tyr, as previously reported (34). This seems unlikely,

however, because under our experimental conditions (Ca^{2+}-free but Mg^{2+}-containing BSS), there was no difference in the p-Tyr content of untreated WI-38 cells in Ca^{2+}-containing and in Ca^{2+}-free BSS (Figs. 6 *G*, 12 *G*, and 7, *A* and *B*, lanes *5*). Therefore, it is more likely that NH_2Cl treatment itself induced p-Tyr dephosphorylation independent of peeling off. These observations also indicate that peeling off requires not only dephosphorylation of p-Tyr, but also an increase in $[Ca^{2+}]_i$. This is supported by the observation that dephosphorylation of p-Tyr by genistein (a tyrosine kinase inhibitor) alone did not induce peeling off, while subsequent application of the microsomal Ca^{2+}-ATPase inhibitor BHQ (26), which increases $[Ca^{2+}]_i$, did (Fig. 8 *C*).

Depletion of microsomal and cytosolic Ca^{2+} by treatment with BHQ plus EGTA prevented both NH_2Cl-induced peeling off and an increase in $[Ca^{2+}]_i$ (Fig. 11 *D*). This observation suggests that NH_2Cl mobilized Ca^{2+} directly or indirectly from intracellular Ca^{2+} storage sites, such as microsomes and sarcoplasmic reticulum. The observations that (*a*) there was no increase in intracellular $InsP_3$ after NH_2Cl treatment in both WI-38 fibroblasts and cardiac myocytes, and (*b*) treatment of isolated rabbit sarcoplasmic reticulum vesicles with various concentrations of NH_2Cl inhibited $^{45}Ca^{2+}$ uptake very rapidly (Fig. 13), indicating that NH_2Cl may have attacked intracellular Ca^{2+} storage sites directly, i.e., NH_2Cl inhibited microsomal Ca^{2+} uptake, and as a result, cytosolic Ca^{2+} increased.

In Ca^{2+}-free medium containing EGTA, NH_2Cl did not induce either an increase in $[Ca^{2+}]_i$ (Fig. 11 *C*) or peeling off (Fig. 10). A possible explanation for this is that even when NH_2Cl induced a transient increase in cytosolic Ca^{2+} by inhibition of microsomal Ca^{2+} uptake, the increased $[Ca^{2+}]_i$ was immediately extruded into the extracellular space when the cells were incubated in Ca^{2+}-free medium containing EGTA. As a result, neither peeling off nor an increase in $[Ca^{2+}]_i$ occurred. This speculation is supported by our observation that the BHQ-induced increase in $[Ca^{2+}]_i$ in WI-38 fibroblasts decreased during subsequent incubation of the cells in Ca^{2+}-free BSS containing BHQ and EGTA (Fig. 11 *D*).

It would be interesting to know whether there is a correlation between p-Tyr dephosphorylation and increased $[Ca^{2+}]_i$. Evidence from some cell systems suggests that an increase in $[Ca^{2+}]_i$ either inhibits tyrosine kinase activity or increases PTPase activity (64, 65). In the present study, however, that p-Tyr dephosphorylation occurred even under conditions in which $[Ca^{2+}]_i$ did not increase (see Figs. 11 *C c* and 12 *H*), suggesting that the NH_2Cl-induced increase in $[Ca^{2+}]_i$ did not cause p-Tyr dephosphorylation. We speculate that NH_2Cl may have attacked focal adhesions and microcosms independently, inducing p-Tyr dephosphorylation and increased $[Ca^{2+}]_i$ at these sites, respectively, and that both of these events are necessary for peeling off. Another possibility is that increase in $[Ca^{2+}]_i$ is also related to the modification of focal adhesion proteins associated with p-Tyr dephosphorylation, because p-Tyr dephosphorylation alone did not change the distribution of other focal adhesion proteins such as integrin or vinculin (Fig. 12, *B* and *D*, and Results), and additional increases in $[Ca^{2+}]_i$ did (Fig. 6 *B* and *D*). Further precise study is needed to reveal the relationship between p-Tyr dephosphorylation and increase in $[Ca^{2+}]_i$ on the disorganization of focal adhesion proteins.

Protective Effect of Fn-Precoating against NH_2Cl-induced Peeling Off

Fn precoating of the substratum prevented both cardiac myocytes and WI-38 cells from NH_2Cl-induced peeling off in a concentration-dependent manner (Figs. 3 and 4), leading us to speculate that peeling off might have been caused by an NH_2Cl-induced alteration of Fn. Exposure of purified human plasma Fn to an HOCl and NH_2Cl generating system (the MPO–H_2O_2-Cl^- system of neutrophils) causes extensive changes in the primary and tertiary structure of Fn (60). Our observation that exposure of Fn to NH_2Cl decreased its specific viscosity supports that finding. NH_2Cl-induced peeling off, however, was also prevented by precoating with Fn that had been pretreated with a high concentration of NH_2Cl, indicating that peeling off is not the result of a conformational alteration of Fn caused by NH_2Cl. Thus, even if the structure of Fn was modified by NH_2Cl, the modification itself did not induce peeling off.

When WI-38 fibroblasts were cultured on Fn-coated dishes, the p-Tyr content of the cells did not change after exposure to NH_2Cl (Fig. 14). This observation suggests that Fn prevented NH_2Cl-induced peeling off via inhibition of an NH_2Cl-induced decrease in the p-Tyr content. It was previously reported that when cells are cultured on an Fn-coated substratum, p-Tyr increases accompanied by an increase in tyrosine kinase activity (6, 18, 21). In our present experiments, the spots bound to antibody against integrin and stress fibers appeared to increase (data not shown), although quantitation was difficult with conventional fluorescence microscopy. The increase in integrin spots and stress fibers might strengthen cell–substratum interactions. The present finding that laminin and collagen types I, III, and IV coating, in contrast to Fn coating, did not prevent NH_2Cl-induced peeling off suggests that these ECM proteins have functionally different roles in cell-to-cell and cell-to-substratum interaction. In rat myocardium, in fact, laminin is found in regions of myocyte–myocyte junctions and Fn is found in regions of myocyte–vascular cell junctions (7).

A Possible Mechanism of NH_2Cl-induced Peeling Off

NH_2Cl causes dephosphorylation of p-Tyr residues of some focal adhesion proteins, and also induces an increase in cytosolic $[Ca^{2+}]_i$ by inhibiting microsomal Ca^{2+} uptake. The increase in $[Ca^{2+}]_i$ in turn triggers actin–myosin interactions, which are followed by cell shrinkage. The role of p-Tyr dephosphorylation may be modification of the conformation and/or activities of focal adhesion proteins, and this may lead to facilitation of cell detachment from the substratum. p-Tyr dephosphorylation alone did not induce peeling off (see Figs. 12 *H*; 8 *B* and *D*), nor did an increase in $[Ca^{2+}]_i$ alone (see Fig. 11 *D b*). Combination of these two events caused peeling off.

Correlation with Stimulated Neutrophil-induced Cytotoxicity

Some evidence suggests that stimulated neutrophils in-

duce morphological injuries in several kinds of tissues (22, 61) and cultured cardiac myocytes (30). Also, cultured normal human keratinocytes (27) and epidermal carcinoma cells (28) detach from the substratum by contact with stimulated neutrophils. The mechanism of neutrophil-induced cytotoxicity, however, has been controversial, because neutrophils produce several kinds of cytotoxic mediators, such as ·OH, NH_2Cl, and elastase, and their effects may overlap. We, therefore, tested various kinds of inhibitors against each mediator to see which particular mediators induce which particular injury. Stimulated neutrophils induced two types of morphological changes in cultured cardiac myocytes: hypercontraction concomitant with balloon formation and peeling off. In the absence of any inhibitors, only hypercontraction was observed, and no peeling off was detected (Fig. 15 *A*). Pretreatment with deferoxamine, DMTU, and α_1-antitrypsin prevented neutrophil-induced hypercontraction but not peeling off (Fig. 15 *B*), and pre-treatment with deferoxamine, DMTU, α_1-antitrypsin, and methionine prevented both types of neutrophil-induced morphological changes (Fig. 15 *C*). These observations indicate that neutrophil-derived ·OH caused hypercontraction, and that neutrophil-derived NH_2Cl caused peeling off. These observations coincide with our previous report showing that xanthine + xanthine oxidase or H_2O_2 caused hypercontraction of cultured cardiac myocytes that was prevented by DMTU and deferoxamine, and that NH_2Cl-induced peeling off was prevented by methionine (41).

The extent of neutrophil-induced peeling off, however, was less than that induced by NH_2Cl (35–48% and 13–15% of cultured cardiac myocytes showed peeling off with 100 μM NH_2Cl and by stimulated neutrophils, respectively) in this study. This is probably because the average concentration of NH_2Cl produced by neutrophils in the medium was <100 μM. What is critical, however, is the NH_2Cl concentration in the vicinity of stimulated neutrophils, but not the average concentration in the medium. In fact, in the present work, the myocytes to which stimulated neutrophils adhered actually showed peeling off and hypercontraction, suggesting that the concentration of NH_2Cl and other oxygen radicals in the vicinity of stimulated neutrophils is high enough to induce these morphological changes in vitro. Because the tissue concentration of NH_2Cl has been estimated to be 200–300 μM by the production of HOCl and NH_4Cl (15), the NH_2Cl concentration we used in this study is physiologically relevant.

In summary, the neutrophil-derived, membrane-permeating oxidant NH_2Cl induced various kinds of cells to detach from the substratum concomitant with cell shrinkage (peeling off). Peeling off was also induced by stimulated neutrophils themselves. NH_2Cl-induced peeling off was prevented by coating the substratum with Fn. Both p-Tyr dephosphorylation and an increase in $[Ca^{2+}]_i$ were mechanisms of NH_2Cl-induced peeling off.

The authors express their thanks to Dr. Mitsuhiro Yokoyama and Dr. Masato Kasuga (Kobe University), Dr. Masao Hayashi and Dr. Keiko Ishikawa (Ochanomizu University) for helpful discussion on this work. We also thank Dr. Tadashi Okamoto, Mr. Hideaki Isoda, Mr. Naoki Kubota, and Ms. Kazue Takahata (Kobe Gakuin University) for helping with the measurement of $[Ca^{2+}]_i$ and the preparation of neutrophils and cultured myocytes. We thank Dr. Keiichi Tanaka and Dr. Norio Muto (Osaka University) for facilitating preparation of this manuscript.

This study was supported in part by the Fund of the Science Research Promotion of Japan Private Promotion Foundation, a grant-in-aid for interdisciplinary research from Kobe Gakuin University, and a grant-in-aid for health science research from Kobe Gakuin University.

Received for publication 22 February 1995 and in revised form 16 June 1995.

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