



Inhibition of degranulation in mast cells attached to a hydrogel through defective microtubule tracts

Atsushi Shiki, Yoshikazu Inoh, Satoru Yokawa, Tadahide Furuno*

School of Pharmacy, Aichi Gakuin University, Nagoya, 464-8650, Japan

ARTICLE INFO

Keywords:

Mast cell
Extracellular matrix
Hydrogel
Focal adhesion
Microtubule
Degranulation

ABSTRACT

Mast cells (MCs) are important effectors of the immediate allergic response. MCs are distributed throughout various tissues and organs, and adhere to extracellular matrix (ECM) with broad stiffness in the body. Here we compared cellular responses following antigen stimulation in MCs on glass-base dishes with and without a hydrogel. We found that an antigen-induced increase in intracellular Ca^{2+} concentration was suppressed slightly in cells on hydrogel-coated dishes compared with those on non-coated dishes, whereas their subsequent degranulation was largely inhibited in cells adherent to the hydrogel. Focusing on focal adhesions (FAs), vinculin was distributed in a dot-like manner at the bottom of resting cells on non-coated dishes but not on hydrogel-coated dishes. According to antigen stimulation, phosphorylation of focal adhesion kinase and additive vinculin accumulation to FAs were promoted in cells on non-coated dishes, but were diminished on hydrogel-coated dishes. Moreover, microtubule reorganization and acetylation (which have important roles in MC degranulation) were also suppressed in activated MCs adherent to the hydrogel. These findings suggest that adhesion to a hydrogel led to failure of composition of functional FAs and microtubule tracts, which resulted in suppression of MC degranulation following antigen stimulation.

1. Introduction

The extracellular matrix (ECM) regulates the proliferation, differentiation, and function of cells through adhesion with resident cells [1,2]. Cell–ECM adhesion complexes provide dynamic links between ECM proteins and the intracellular cytoskeleton thanks to flexible engagement, clustering, and turnover.

Integrins are major cellular adhesion molecules and are concentrated prominently in focal adhesions (FAs), where cells “sense” the chemical and mechanical properties of their environment. The extracellular domains of integrins bind to ECM ligands, and the cytoplasmic domain interacts with adaptor proteins such as talin, paxillin, vinculin, and focal adhesion kinase (FAK) to link with the cytoskeleton.

Cell–ECM adhesion sites are highly versatile “mechanosensing” systems and enable cells to monitor their adhesive states. Hence, cells can respond rapidly to changes in mechanical tension or shear stress by modulation of the adhesion and migration of cells [3].

An increase in force loading and mechanical tension on cell–ECM adhesion sites induces recruitment of adaptor proteins and FA maturation. Engagement of integrins with the ECM promotes paxillin phosphorylation, and the phosphorylated paxillin molecules contribute to recruitment of FAK and vinculin as scaffold proteins [4]. FAK

phosphorylation generates a binding site for the SH2 domain of Src tyrosine kinase. The FAK/Src complex phosphorylates assembled proteins to FAs and has a role in “mechanotransduction”.

Vinculin has three functional domains (head, neck, tail) and exists in two conformational forms in cells (open activation and closed auto-inhibition) [5–8]. In the closed form, the tight binding of the head and tail domains mask phosphorylation sites and hinder the ability to bind target proteins. Force loading and phosphorylation have been proposed to shift the vinculin conformation from a closed auto-inhibition form to an open activation form. Vinculin can bind to talin, paxillin, and F-actin in an open activation form and regulate transmission of mechanical signals from the ECM to the cytoskeletal machinery. Thus, force across vinculin stimulates the protein assembly and FA maturation, indicating that vinculin plays a key part in the transduction of extracellular mechanical stimuli to cytoskeletal reorganization. F-actin anchored to FAs by vinculin interacts with microtubules through fascin, an actin- and microtubule-binding protein [9], suggesting that FAs are also involved in the polarity and motility of cells.

Mast cells (MCs) are important effector cells of the immediate allergic response. MCs secrete allergic mediators such as histamine and serotonin prestored in intracellular granules [10]. These cells are distributed throughout various tissues and organs, some of which are

* Corresponding author School of Pharmacy, Aichi Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya, 464-8650, Japan.

E-mail address: furuno@dpc.agu.ac.jp (T. Furuno).

<https://doi.org/10.1016/j.yexcr.2019.05.019>

Received 2 February 2019; Received in revised form 13 May 2019; Accepted 15 May 2019

0014-4827/ © 2019 Published by Elsevier Inc.

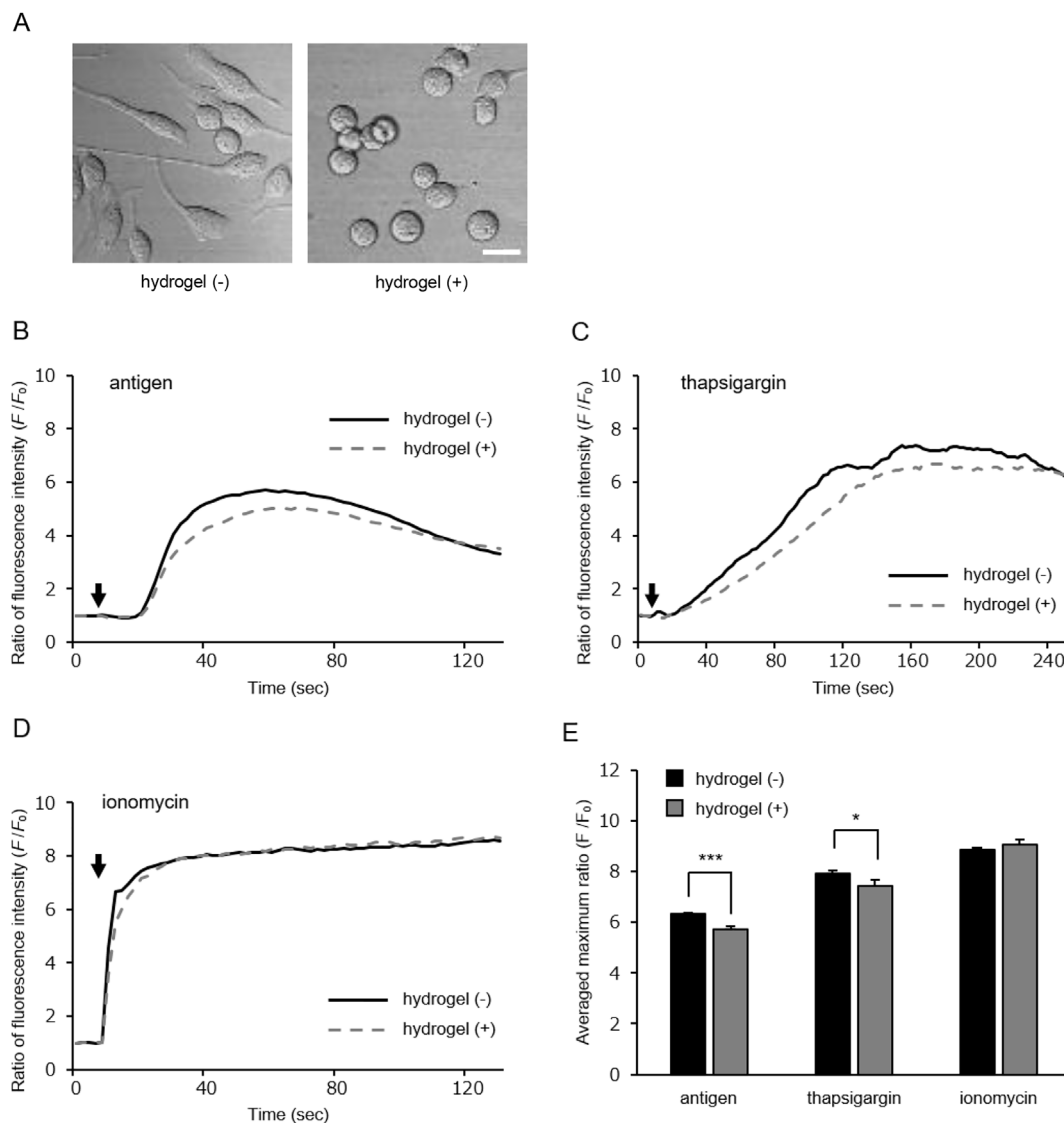


Fig. 1. $[Ca^{2+}]_i$ increase in activated RBL-2H3 cells following antigen stimulation.

(A) Differential interference contrast (DIC) images of RBL-2H3 cells in dishes coated with and without the hydrogel. Scale bar = 20 μm . (B–D) Time courses of $[Ca^{2+}]_i$ after addition of antigen (DNP-BSA; 50 ng/mL) (B), thapsigargin (50 nM) (C), and ionomycin (1 μM) (D) in cells on glass-base dishes coated with (dotted lines) and without (solid lines) the hydrogel. The averaged ratio data (F/F_0), which were calculated by dividing Fluo-4 fluorescence intensity (F) after addition of stimulants by the fluorescence intensity (F_0) at resting level ($t = 0$), of 50 cells are presented. Arrows indicate the time when the stimulants were added. (E) Comparison of maximum ratio values of Fluo 4 fluorescence intensity after stimulant addition between cells on glass-base dishes coated with (gray boxes) and without (black boxes) the hydrogel. Results are the mean $\pm$ SEM from 50 cells. * $P < 0.05$ and *** $P < 0.001$ by Student's t -test.

influenced by mechanical stretch (e.g., heartbeat, respiration) and adhere to ECM proteins (e.g., fibronectin, vitronectin, laminin) by binding to their adhesion molecules, including integrins [11,12].

Adhesion of MCs to ECM proteins is known to enhance MC degranulation by crosslinking to high-affinity immunoglobulin (Ig)E receptors (Fc ϵ RI) on their plasma membrane [13]. In turn, MC activation amplifies their adhesion to ECM proteins [14]. Fc ϵ RI crosslinking by multivalent antigen initiates activation of cascades of Src family tyrosine kinases, including Lyn and Fyn [10]. Phosphorylated Lyn induces phosphorylation of Syk tyrosine kinase, which subsequently activates several adaptor proteins, including phospholipase $C\gamma$ (PLC γ), which generates inositol-1,4,5-trisphosphate (IP_3) by hydrolysis of phosphatidylinositol-4,5-bisphosphate and diacylglycerol (DAG). IP_3 binds to its receptor (IP_3R) on the endoplasmic reticulum (ER) membrane and triggers Ca^{2+} release from the ER. The decreased Ca^{2+} concentration in the ER subsequently induces extracellular Ca^{2+} influx into the cytoplasm via stromal interaction molecule–Orai interaction. The lasting

increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), as well as activation of protein kinase C (PKC) by DAG, is essential for MC degranulation.

In addition, degranulation is accompanied by reorganization of the cytoskeleton, which leads to membrane ruffling and cell spreading. Movement of secretory granules toward the plasma membrane, where they fuse to release allergic mediators, is dependent on the microtubule tract [15].

Kinesin-1/Slp3/Rab27b and dynein/RILP/Rab12 complexes have been reported to have roles in the anterograde and retrograde transport of secretory granules along microtubules in MCs, respectively [16,17]. Post-translational modifications of tubulins, including acetylation, de-tyrosination, glutamylation, and glycylation, affect not only the dynamics but also the reorganization and interaction of microtubules with motor proteins [18–20]. Acetylation at Lys40 of α -tubulin, for instance, does not affect microtubule structure, but it protects long-lived microtubules and promotes the binding and transport of kinesin-1 [21–23].

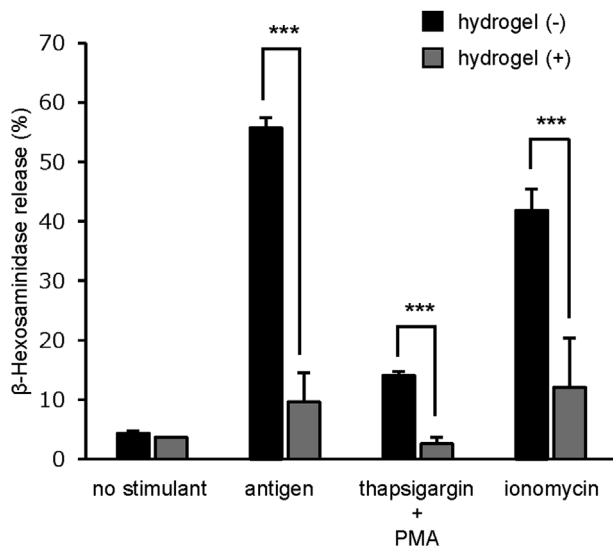


Fig. 2. Degranulation of RBL-2H3 cells following antigen stimulation. Percentage of released β -hexosaminidase to the total content after addition of antigen (DNP-BSA; 50 ng/mL), thapsigargin (50 nM) + PMA (50 ng/mL), or ionomycin (1 μ M) for 30 min in cells adherent to plates coated with (gray boxes) and without (black boxes) the hydrogel. Results are the mean $\pm$ SEM from three independent experiments. *** P < 0.001 by Student's t -test.

Recently, we showed that microtubule acetylation was promoted according to antigen stimulation by western blotting and immunocytochemistry, and played an important part in MC degranulation [24].

Here, we studied the MC response on a hydrogel, focusing on FA assemblies and the cytoskeleton tract. The hydrogel used in the present study comprised thiol-modified hyaluronic acid and thiol-modified gelatin with the crosslinking reagent disulfide-containing polyethylene glycol diacrylate [25]. We found that accumulation of vinculin to FAs was inhibited in resting MCs on a hydrogel. According to antigen stimulation, microtubule acetylation and microtubule-organizing center (MTOC) formation was impaired and, thereafter, degranulation was also suppressed significantly in MCs on the hydrogel. Our results might provide novel information on the molecular mechanism in relation to

cellular exocytosis and adhesion to ECM.

2. Materials and methods

2.1. Materials

Mouse *anti*-dinitrophenyl (DNP) IgE (IgE-53-569) was kindly provided by Professor T. Kishimoto (Osaka University, Suita, Japan). DNP-conjugated bovine serum albumin (DNP-BSA) was prepared using methods described previously [26]. In the present study, seven DNP groups, on average, were conjugated with a single BSA molecule [27–29]. Thapsigargin and ionomycin were purchased from Wako Pure Chemical Industries (Osaka, Japan). Phorbol-12-myristoyl-13-acetate (PMA) and paclitaxel were obtained from Sigma–Aldrich (Saint Louis, MO, USA).

2.2. Hydrogel preparation

The hydrogel was prepared using a Hystem[®]-C hydrogel kit (ESI BIO, Alameda, CA, USA). According to the technical datasheet, Hystem[®]-C hydrogel can display compliance to stresses between a few hundred to a few thousand Pa. In brief, after mixing 1% Glycosil[®] (thiol-modified hyaluronic acid) and 1% Gelin-S[®] (thiol-modified gelatin) in a 1:1 vol ratio, 2% PEGSSDA[®] (disulfide-containing polyethylene glycol diacrylate) was added to the mixture in a 1:4 vol ratio. The solution was applied to 35-mm glass-base dishes (AGC, Tokyo, Japan) and 96-well culture plates (Corning, Corning, NY, USA) and maintained at room temperature until gelation.

2.3. Cell culture

The rat MC line RBL-2H3 (the line used most widely as a model for MCs) was cultured in an atmosphere of 5% CO₂ at 37 °C in minimum essential medium (MEM) (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) (Roche, Mannheim, Germany) [27–30].

2.4. $[Ca^{2+}]_i$ measurement

RBL-2H3 cells were cultured on glass-base dishes coated with or without the hydrogel overnight. The following day, cells were loaded with Fluo 4-AM (1 μ M) (Dojindo, Kumamoto, Japan) and *anti*-DNP IgE

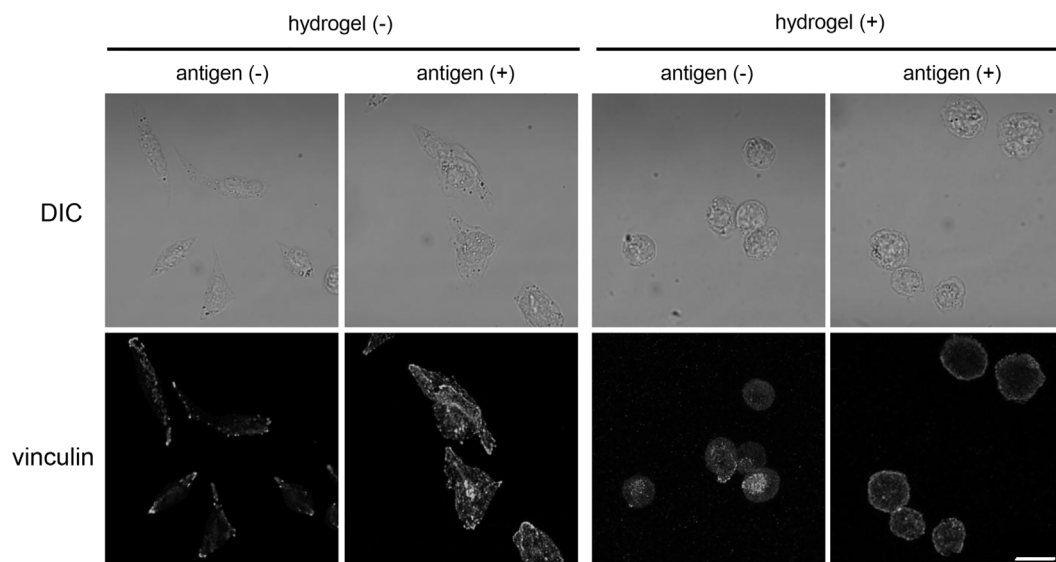


Fig. 3. Distribution of vinculin in RBL-2H3 cells following antigen stimulation. Confocal fluorescence images with vinculin staining in cells cultured on glass-base dishes coated with and without the hydrogel at an adhesive plane. Cells were fixed in 2% formaldehyde before and 10-min after antigen stimulation. Scale bar = 20 μ m.

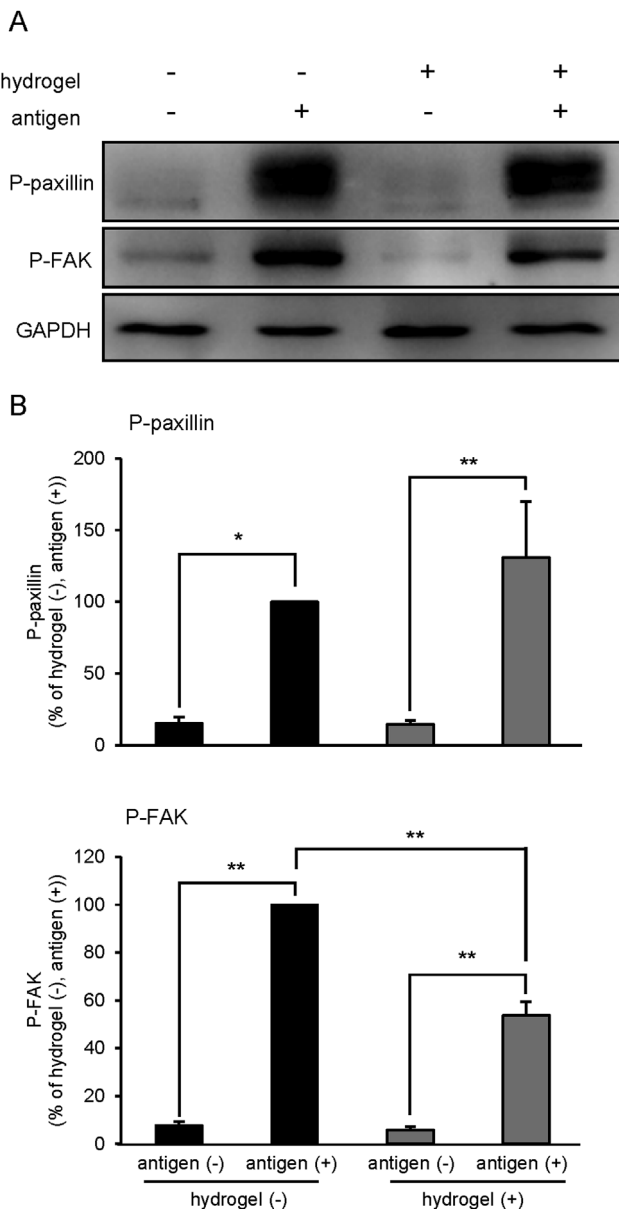


Fig. 4. Phosphorylation of paxillin and FAK in RBL-2H3 cells following antigen stimulation.

(A) Phosphorylation levels of paxillin (P-paxillin) and FAK (P-FAK) before and 10-min after antigen (DNP-BSA; 50 ng/mL) stimulation in cells on glass-base dishes coated with and without the hydrogel measured by western blotting. (B) Relative phosphorylation levels to antigen-stimulated cells adherent to glass (as 100%) are presented after controlling for protein loading. GAPDH served as the control. Data are representative of three independent experiments. Results are the mean $\pm$ SE from > 3 independent experiments. $^{***}P < 0.01$ by Tukey-Kramer's *t*-test.

(1:5000 dilution) for 30 min at 37 °C, and then washed twice with Hepes buffer [10 mM HEPES (pH 7.2), 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl₂, 1 mM CaCl₂, 0.1% glucose, 0.1% BSA, and 0.01% sulfapyrazone]. Preparations were viewed on a confocal laser scanning microscope (LSM-800; Zeiss, Oberkochen, Germany). During experiments, the temperature of the observation chambers was maintained at 37 °C. Fluorescence intensity was measured by ZEN software (Zeiss). The ratio data (F/F_0) were calculated by dividing fluorescence intensity (F) after addition of stimulants by the fluorescence intensity (F_0) at resting level ($t = 0$) [29].

2.5. β -Hexosaminidase assay

Degranulation of RBL-2H3 cells was monitored by measuring the activity of the granule-stored enzyme β -hexosaminidase secreted into cell supernatants. Cells were plated in 96-well plates coated with or without the hydrogel overnight. The following day, cells were sensitized with *anti*-DNP IgE (1:5000) for 30 min at 37 °C, monolayers were washed with Hepes buffer, and incubated with stimulants for 30 min at 37 °C. Following incubation, supernatant aliquots were transferred to 96-well plates (20 μ L/well) and incubated with 20 μ L substrate solution [2 mM *p*-nitrophenyl-N-acetyl- β -D-glucosaminide (Sigma-Aldrich) in 100 mM citrate buffer (pH 4.5)] for 1 h at 37 °C. After terminating the reaction using 167 mM Na₂CO₃-NaHCO₃ buffer (160 μ L), absorbance at 405 nm was measured using a microplate reader (680; Bio-Rad Laboratories, Hercules, CA, USA). Release activity relative to total cellular β -hexosaminidase content was determined by disrupting cells with 0.1% Triton X-100 [28–31]. Each assay was undertaken in triplicate.

2.6. Immunostaining

RBL-2H3 cells were seeded on glass-base dishes coated with or without the hydrogel overnight. The following day, cells were sensitized with *anti*-DNP IgE (1:5000) for 30 min at 37 °C, and monolayers washed with Hepes buffer and then stimulated with DNP-BSA (50 ng/mL) for 10 min at 37 °C. Cells were fixed in 2% paraformaldehyde for 15 min, permeabilized in 0.3% Triton X-100 for 30 min, and blocked in 1% BSA for 30 min.

In single staining, cells were probed for 1 h with mouse *anti*-vinculin antibody (1:400; Sigma-Aldrich) and incubated for 1 h with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000; Thermo Fischer Scientific, Waltham, MA, USA). In double staining, cells were probed for 1 h with mouse anti-acetylated- α -tubulin antibody (K40) (1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated for 1 h with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000) and 0.1 μ g/mL 4,6-diamidino 2-phenylindole (DAPI) (Sigma-Aldrich). In triple staining, cells were probed for 1 h with mouse *anti*- α -tubulin antibody (1:400; Sigma-Aldrich) and incubated for 1 h with Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000), 13.2 nM rhodamine X-conjugated phalloidin (Wako Pure Chemical Industries) and 0.1 μ g/mL DAPI [32].

Fluorescence intensity was measured and analyzed by ZEN software. α -tubulin accumulation in MTOCs was analyzed by a procedure reported recently [24]. In brief, after the fluorescent signal for α -tubulin had been measured in circles with radii of 1 μ m and 5 μ m in MTOCs and cytoplasm, respectively, in a single cell, the ratio of fluorescence intensity in MTOCs to that in cytoplasm was calculated. Quantification of acetylated microtubules was done by measuring the average fluorescence intensity within the cell periphery.

2.7. Western blotting

Cultured RBL-2H3 cells (5×10^5 cells/well) were seeded in 35-mm plates coated with or without the hydrogel. The following day, cells were sensitized with *anti*-DNP IgE (1:5000) for 30 min at 37 °C, and monolayers were washed with Hepes buffer and stimulated with DNP-BSA (50 ng/mL) for 10 min at 37 °C. Thereafter, cells were lysed with cold lysis buffer [20 mM HEPES (pH 7.9), 0.1% NP-40, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM Na₃VO₄, 10 μ g/mL phenylmethylsulfonyl fluoride, 10 μ g/mL leupeptin, and 10% glycerol]. Lysates were maintained on ice for 30 min, followed by centrifugation at 15,000 $\times$ g for 10 min at 0 °C. The resulting supernatants were solubilized by treatment with Laemmli buffer at 100 °C for 5 min. Subsequently, proteins were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes with an

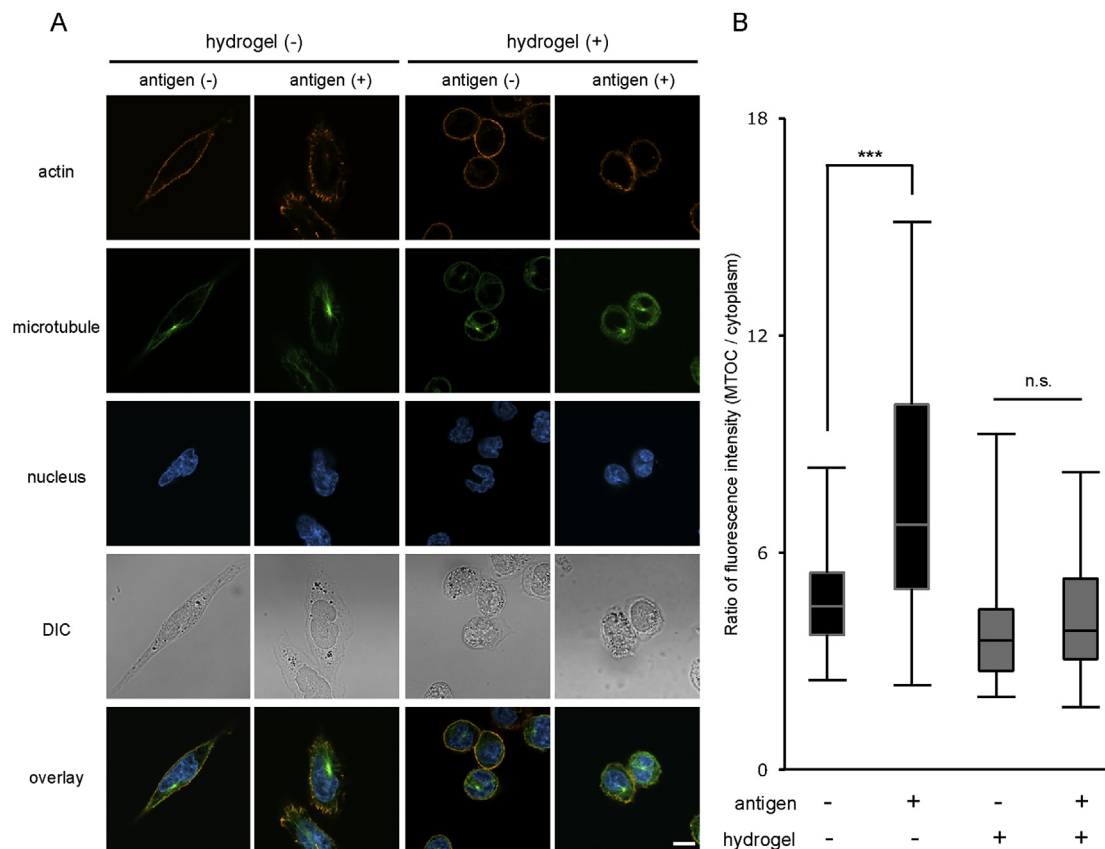


Fig. 5. Inhibition of microtubule reorganization following antigen stimulation in RBL-2H3 cells adherent to the hydrogel.

(A) Confocal fluorescence images with triple staining for actin (red), α -tubulin (green) and nuclei (blue) in cells on glass-base dishes coated with and without the hydrogel before and 10-min after antigen (DNP-BSA; 50 ng/mL) stimulation. Scale bar = 20 μ m. (B) Ratio of fluorescence intensity in MTOCs to that in cytoplasm of cells on glass-base dishes coated with and without the hydrogel before and 10-min after antigen (DNP-BSA; 50 ng/mL) stimulation. *** P < 0.001 by Tukey–Kramer's test. n.s.; not significant.

electroblotter (Bio-Rad Laboratories). After blockade with 5% BSA, PVDF membranes were probed with rabbit *anti-phospho-FAK* (Y397) (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit *anti-phospho-paxillin* (Y118) (1:1000; Cell Signaling Technology), or rabbit *anti-glyceraldehyde 3-phosphate dehydrogenase* (GAPDH) antibody (1:5000; Sigma–Aldrich) at 4 °C overnight. Subsequently, treatment with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:2000; Cell Signaling Technology) or HRP-labeled goat anti-mouse IgG antibody (1:2000; Cell Signaling Technology) was done. Immunoreactivity was detected using enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK) with a LAS-4000 imager (Fujifilm, Tokyo, Japan) and analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.8. Statistical analyses

The Student's *t*-test and Tukey–Kramer's test were used to compare group differences. Results were considered significant at P < 0.05.

3. Results

3.1. $[Ca^{2+}]_i$ increase and degranulation of RBL-2H3 cells

First, we compared cell shape on glass-base dishes coated with and without the hydrogel that shows stresses between a few hundred to a few thousand Pa. Whereas RBL-2H3 cells were spread on glass-base dishes without the hydrogel, their shape was round on hydrogel-coated dishes (Fig. 1A), as reported for other cell types [33,34]. First we measured the $[Ca^{2+}]_i$ increase in individual RBL-2H3 cells adherent on

glass-base dishes coated with and without the hydrogel following addition of antigen, a sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA) inhibitor (thapsigargin) and a Ca^{2+} ionophore (ionomycin) using confocal laser scanning microscopy.

Antigen induced a rapid and lasting $[Ca^{2+}]_i$ increase after a short lag-period, whereas thapsigargin led to a gradual and maintained $[Ca^{2+}]_i$ increase in RBL-2H3 cells on glass (Fig. 1B and C), as reported previously [35]. As expected, ionomycin increased the $[Ca^{2+}]_i$ suddenly in RBL-2H3 cells (Fig. 1D). Comparing the averaged maximum ratio of fluorescence intensity, the amount of $[Ca^{2+}]_i$ increase was suppressed slightly in cells stimulated with antigen and thapsigargin, but not ionomycin, on hydrogel-coated dishes (Fig. 1E).

Next, we measured degranulation of RBL-2H3 cells 30 min after addition of stimulants. Thapsigargin alone did not induce degranulation sufficiently, so co-stimulation of thapsigargin with the PKC activator PMA (thapsigargin + PMA) was used to detect β -hexosaminidase release. Interestingly, unlike the $[Ca^{2+}]_i$ increase, degranulation of RBL-2H3 cells on the hydrogel was inhibited greatly after addition of antigen, thapsigargin + PMA, or ionomycin (Fig. 2). These results suggested that adhesion to the hydrogel did not affect the $[Ca^{2+}]_i$ increase largely but showed considerable inhibition of degranulation in RBL-2H3 cells after Fc ϵ RI-dependent and -independent stimulation.

3.2. Vinculin accumulation to FAs in RBL-2H3 cells

Degranulation rather than a $[Ca^{2+}]_i$ increase was largely inhibited in activated RBL-2H3 cells on the hydrogel, which originally had a round shape, so we considered that FA maturation and/or cytoskeletal reorganization were involved in this diminished degranulation. We

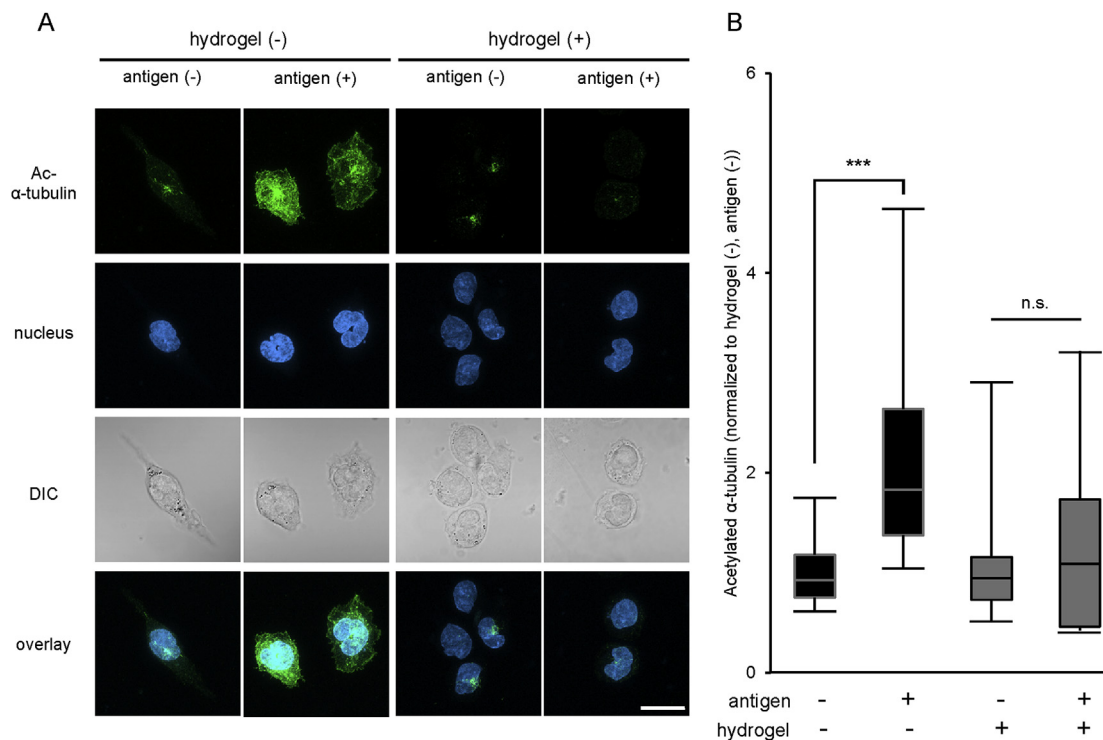


Fig. 6. Inhibition of microtubule acetylation following antigen stimulation in RBL-2H3 cells adherent to the hydrogel.

(A) Z-stack images of confocal fluorescence microscopy with double staining of acetylated α -tubulin (Ac- α -tubulin) (green) and nuclei (blue) in cells on glass-base dishes coated with and without the hydrogel before and 10-min after antigen (DNP-BSA; 50 ng/mL) stimulation. Scale bar = 20 μ m. (B) Normalized fluorescence intensity of acetylated α -tubulin in cells on glass-base dishes coated with and without the hydrogel before and 10-min after antigen (DNP-BSA; 50 ng/mL) stimulation. *** P < 0.001 by Tukey–Kramer's test. n.s.; not significant.

observed the intracellular distribution of vinculin (which is recruited to FAs and transduces extracellular mechanical stimuli to cytoskeletal reorganization) in resting RBL-2H3 cells on glass-base dishes coated with and without the hydrogel. Vinculin showed a dot-like distribution on the bottom of cells on non-coated dishes, but was distributed in the entirety of cytoplasm in cells on hydrogel-coated dishes (Fig. 3). Vinculin is involved in the connection between the ECM and cytoskeletal machinery in FAs, so this result suggested that formation of FA assemblies was defective in resting cells attached to the hydrogel.

3.3. Phosphorylation of FA proteins in RBL-2H3 cells

Paxillin and FAK, both of which are intracellular proteins composed of FAs, are known to be phosphorylated in MCs according to antigen stimulation [36,37]. We measured phosphorylation of paxillin and FAK in antigen-stimulated RBL-2H3 cells on glass-base dishes coated with and without the hydrogel. Phosphorylation of paxillin and FAK in resting cells on hydrogel-coated dishes was similar to that on non-coated dishes (Fig. 4). Following cellular activation with antigen, the phosphorylation of paxillin and FAK was enhanced significantly in cells on non-coated dishes and hydrogel-coated dishes. Phosphorylation of paxillin, an integrin-binding protein, was promoted in stimulated cells on glass-base dishes coated with and without the hydrogel. However, phosphorylation of FAK, a recruited protein to FAs by phosphorylated paxillin, was weaker in stimulated cells on hydrogel-coated dishes than those on non-coated dishes. In accordance with these results, the additive accumulation of vinculin to FAs according to antigen stimulation was observed in cells on non-coated dishes, but not on hydrogel-coated dishes (Fig. 3). These data suggested that paxillin phosphorylation was evoked similarly beneath the plasma membrane in contact with glass and the hydrogel according to antigen stimulation but, thereafter, FAK phosphorylation and vinculin recruitment were impaired in cells on the hydrogel.

3.4. Microtubule reorganization in RBL-2H3 cells

Defective re-assembly of FAs induced by antigen in cells attached to the hydrogel was considered to affect the cytoskeletal protein network so, next, we studied the intracellular distribution of the cytoskeleton in MCs. As described previously [38], F-actin was localized on the periphery of RBL-2H3 cells in dishes coated with and without the hydrogel (Fig. 5A). The stronger ruffling of plasma membranes derived from degranulation occurred in cells on non-coated dishes than on hydrogel-coated dishes. A filamentous microtubule network from MTOCs was observed clearly in the cytoplasm of resting cells. Microtubules are known to be re-organized after antigen stimulation by accumulation of α -tubulins to MTOCs [24]. We compared the microtubule reorganization in cells on dishes coated with and without the hydrogel. Microtubule reorganization was accelerated significantly according to antigen stimulation in cells on non-coated dishes but not on hydrogel-coated dishes (Fig. 5B).

α -tubulin acetylation in microtubules has been reported to induce microtubule stabilization and acceleration of cargo transport [21–23]. Recently, we found that promotion of microtubule acetylation played an important part in MC degranulation [24]. Hence, we measured microtubule acetylation in individual cells on dishes coated with and without the hydrogel (Fig. 6A). Microtubule acetylation was promoted according to antigen stimulation in cells on non-coated dishes, but not on hydrogel-coated dishes (Fig. 6B). These results suggested that the suppressive effect of MC degranulation on hydrogel-coated dishes was involved in defective microtubule tracts.

4. Discussion

In this study, we compared cellular responses following antigen stimulation in dishes coated with and without the hydrogel. Cell-deposited native tissues were estimated to range in stiffness from ~ 0.1 to

~1000 kPa [39], which were intermediate between the hydrogel used here (a few hundred to a few thousand Pa) and glass or plastic dishes (~10⁸ Pa). When RBL-2H3 cells were two-dimensionally cultured on the surface of glass or hydrogel under the same condition (in an atmosphere of 5% CO₂ at 37 °C in MEM supplemented with 10% FCS) overnight, cell shape was quite different between the cultured dishes; cells were spread on glass, while they were round on the hydrogel. We considered that the difference in cell shape was derived from the different formation of FA assemblies and cytoskeleton organization. Actually, vinculin was distributed in a dot-like manner in the bottom of resting cells attached to glass but not to the hydrogel. We are considering that the FA assembly formation in cells on glass without hydrogel was mainly due to ECM proteins such as fibronectin in FCS included in culture medium. While culturing RBL-2H3 cells, ECM proteins in FCS were adsorbed onto the glass surface and then the cells were attached to the adsorbed ECM proteins by integrins [40].

FA-composing proteins have been observed to be colocalized with FcεRI aggregates following antigen stimulation in RBL-2H3 cells [41]. Hence, we speculated that defective FA assemblies and/or cytoskeleton organization might affect the formation of FcεRI crosslinks on the plasma membrane after antigen stimulation and, subsequently, influence the response of MCs attached to the hydrogel. Unexpectedly, the antigen-induced [Ca²⁺]_i increase was suppressed only slightly in cells attached to the hydrogel. The [Ca²⁺]_i increase in cells on the hydrogel after addition of thapsigargin (which leads to Ca²⁺ leakage from the ER by inhibiting SERCA) was also suppressed slightly. The ionomycin-induced [Ca²⁺]_i increase was not suppressed in cells on the hydrogel, suggesting that the defective FA assemblies did not affect the [Ca²⁺]_i increase mediated by FcεRI crosslinking greatly, and that slight inhibition of the [Ca²⁺]_i increase was due to the suppressive effect of defective FA assemblies on store-operated Ca²⁺ entry channels. On the other hand, unlike the [Ca²⁺]_i increase, the subsequent degranulation of cells on the hydrogel was largely inhibited according to their stimulants. To investigate whether gelatin included in the hydrogel affected the cell response, we compared the [Ca²⁺]_i increase and degranulation of RBL-2H3 cells on glass coated with and without collagen. No significant difference in the [Ca²⁺]_i increase and degranulation between the presence and absence of collagen was found (data not shown), suggesting that gelatin in the hydrogel little influenced the cell response.

Paxillin is an integrin-binding protein and recruits FAK and vinculin for FA assemblies. Defective accumulation of vinculin to the bottom of resting cells on the hydrogel showed that functional FA assemblies were not formed in those cells. Adherence to the ECM as well as crosslinking of FcεRI has been reported to induce tyrosine phosphorylation of paxillin and FAK in RBL-2H3 cells [36,37]. Here, in resting cells, phosphorylation of paxillin and FAK did not show a significant difference between cells adherent to dishes coated with and without the hydrogel. According to antigen stimulation, phosphorylation of paxillin and FAK was accelerated significantly in cells on dishes with and without the hydrogel. However, phosphorylation of FAK, but not paxillin, was lower in cells on hydrogel-coated dishes than those on non-coated dishes. Additive accumulation of vinculin according to antigen stimulation, which occurred in cells on glass, was not detected in cells attached to the hydrogel. These findings suggested that phosphorylation of the paxillin that binds to integrins was induced similarly in cells adherent to glass and the hydrogel after antigen stimulation, but that its downstream events in FAs (including FAK phosphorylation and vinculin accumulation) were suppressed in cells attached to the hydrogel.

Though no great difference in intracellular distribution of actin filament and level of [Ca²⁺]_i increase according to antigen stimulation was shown in cells on dishes with and without the hydrogel, the plasma membrane ruffling induced by antigen was weaker in cells on hydrogel-coated dishes. The plasma membrane ruffling was reported to require phospholipase D (PLD) activity in RBL-2H3 cells [42]. The weak membrane ruffling might be due to impaired promotion of PLD activity

after antigen stimulation in cells on hydrogel.

Moreover, FAK phosphorylation has been reported to stimulate microtubule acetylation through Ras homolog gene family, member A (RhoA) activation [43]. Microtubule acetylation upregulates intracellular transport of granules by regulating traffic of kinesin motors [23,44]. A recent study showed that collaboration between paxillin and FAK was important for modulation of microtubule acetylation, and that paxillin could not induce microtubule acetylation without FAK activity [45]. Adhesion to the ECM and stimulation with antigen induce FAK phosphorylation mediated by different pathways [46]. Therefore, impaired phosphorylation of FAK according to antigen stimulation was considered to cause the defective microtubule tract mediated by insufficient microtubule acetylation. Reports have shown a dynamic relationship between FAs and microtubules [47–49]. FAs have been reported to act as preferred sites of the nucleation, polymerization, and stabilization of microtubules in Swiss 3T3 fibroblasts [47]. Recently, microtubules were found to not only induce FA disassembly and turnover for cell migration, but also to establish a pathway for delivery of exocytic granules near FAs [48,49].

We showed that promotion of microtubule acetylation and reorganization following antigen stimulation in cells on glass was inhibited in cells on a hydrogel. Insufficient modification of microtubules was probably due to insufficient FA formation in cells on the hydrogel. We hypothesize that: (i) adhesion to the hydrogel disturbed formation of functional FA assemblies; (ii) dysfunctional FAs impaired antigen-induced microtubule modification such as acetylation; (iii) a defective microtubule tract through motor proteins led to inhibition of degranulation of RBL-2H3 cells.

5. Conclusions

We conclude that MC degranulation was small significantly by defective microtubule tracts based on insufficient FA formation on a hydrogel compared with glass and plastic widely used as a cell culture substrate. We have here expressed that MC function was reduced and suppressed on a hydrogel from the view that a glass or plastic is standard. However, the expression that MC function was increased and enhanced on a glass or plastic substrate may be more suitable because native tissue stiffness is estimated to be intermediate between the hydrogel and glass used here. To understand which expression is appropriate, further experiments are required under the ECM stiffness close to native tissues.

Funding

This study was supported, in part, by Japan Society for the Promotion of Science (Japan) KAKENHI (Grant Numbers: 17K08255 to YI and 17K07374 to TF).

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgments

We thank Professor T. Kishimoto (Osaka University, Osaka, Japan) for supplying *anti*-DNP monoclonal IgE. We thank Arshad Makhadmeh, PhD, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

References

- [1] J.K. Mouw, G. Ou, V.M. Weaver, Extracellular matrix assembly: a multiscale deconstruction, *Nat. Rev. Mol. Cell Biol.* 15 (2014) 771–785.
- [2] F. Kai, H. Laklai, V.M. Weaver, Force matters: biomechanical regulation of cell invasion and migration in disease, *Trends Cell Biol.* 26 (2016) 486–497.

- [3] V.P. Hytönen, B. Wehrle-Halle, Mechanosensing in cell-matrix adhesions: converting tension into chemical signals, *Exp. Cell Res.* 343 (2016) 35–41.
- [4] A.M. López-Colomé, I. Lee-Rivera, R. Benavides-Hidalgo, E. López, Paxillin: a crossroad in pathological cell migration, *J. Hematol. Oncol.* 10 (2017) 50.
- [5] A. Carisey, R. Tsang, A.M. Greiner, N. Nijenhuis, N. Heath, A. Nazgiewicz, R. Kemkemer, B. Derby, J. Spatz, C. Ballestrem, Vinculin regulates the recruitment and release of core focal adhesion proteins in a force-dependent manner, *Curr. Biol.* 23 (2013) 271–281.
- [6] P. Atherton, B. Stutchbury, D. Jethwa, C. Ballestrem, Mechanosensitive components of integrin adhesions: role of vinculin, *Exp. Cell Res.* 343 (2016) 21–27.
- [7] W.H. Goldmann, Role of vinculin in cellular mechanotransduction, *Cell Biol. Int.* 40 (2016) 241–256.
- [8] J.L. Bays, K.A. DeMali, Vinculin in cell-cell and cell-matrix adhesions, *Cell. Mol. Life Sci.* 74 (2017) 2999–3009.
- [9] G. Villari, A. Jayo, J. Zanet, B. Fitch, B. Serrels, M. Frame, B.M. Stramer, B.T. Goult, M. Parsons, A direct interaction between fascin and microtubules contributes to adhesion dynamics and cell migration, *J. Cell Sci.* 128 (2015) 4601–4614.
- [10] J. Kalesnikoff, S.J. Galli, New developments in mast cell biology, *Nat. Immunol.* 9 (2008) 1215–1223.
- [11] M. Columbo, B.S. Bochner, G. Marone, Human skin mast cells express functional β_1 integrins that mediate adhesion to extracellular matrix proteins, *J. Immunol.* 154 (1995) 6058–6064.
- [12] S. Kraft, T. Fleming, J.M. Billingsley, S.Y. Lin, M.H. Jouvin, P. Storz, J.P. Kinet, Anti-CD63 antibodies suppress IgE-dependent allergic reactions in vitro and in vivo, *J. Exp. Med.* 201 (2005) 385–396.
- [13] M.M. Hamawy, C. Oliver, S.E. Mergenhagen, R.P. Siraganian, Adherence of rat basophilic leukemia (RBL-2H3) cells to fibronectin-coated surfaces enhances secretion, *J. Immunol.* 149 (1992) 615–621.
- [14] H.L. Thompson, P.D. Burbelo, D.D. Metcalfe, Regulation of adhesion of mouse bone marrow-derived mast cells to laminin, *J. Immunol.* 145 (1990) 3425–3431.
- [15] K. Nishida, S. Yamasaki, Y. Ito, K. Kabu, K. Hattori, T. Tezuka, H. Nishizumi, D. Kitamura, R. Goitsuka R, R.S. Geha, T. Yamamoto, T. Yagi, T. Hirano, Fc ϵ RI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane, *J. Cell Biol.* 170 (2005) 115–126.
- [16] I. Munoz, L. Danelli, J. Claver, N. Goudin, M. Kurowska, I.K. Madera-Salcedo, J.D. Huang, A. Fischer, C. González-Espinoza, G. de Saint Basile, U. Blank, G. Ménasché, Kinesin-1 controls mast cell degranulation and anaphylaxis through PI3K-dependent recruitment to the granular Slp3/Rab27b complex, *J. Cell Biol.* 215 (2016) 203–216.
- [17] A. Efergan, N.P. Azouz, O. Klein, K. Noguchi, M.E. Rothenberg, M. Fukuda, R. Sagi-Eisenberg, Rab12 regulates retrograde transport of mast cell secretory granules by interacting with the RILP-dynein complex, *J. Immunol.* 196 (2016) 1091–1101.
- [18] C. Janke, The tubulin code: molecular components, readout mechanisms, and functions, *J. Cell Biol.* 206 (2014) 461–472.
- [19] Y. Song, S.T. Brady, Post-translational modifications of tubulin: pathways to functional diversity of microtubules, *Trends Cell Biol.* 25 (2015) 125–136.
- [20] D. Wloga, E. Joachimiak, H. Fabczak, Tubulin post-translational modifications and microtubule dynamics, *Int. J. Mol. Sci.* 18 (2017) 2207.
- [21] S.C. Howes, G.M. Alushin, T. Shida, M.V. Nachury, E. Nogales, Effects of tubulin acetylation and tubulin acetyltransferase binding on microtubule structure, *Mol. Biol. Cell* 25 (2014) 257–266.
- [22] D. Portran, L. Schaedel, Z. Xu, M. Théry, M.V. Nachury, Tubulin acetylation protects long-lived microtubules against mechanical ageing, *Nat. Cell Biol.* 19 (2017) 391–398.
- [23] N.A. Reed, D. Cai, T.L. Blasius, G.T. Jih, E. Meyhofer, J. Gaertig, K.J. Verhey, Microtubule acetylation promotes kinesin-1 binding and transport, *Curr. Biol.* 16 (2006) 2166–2172.
- [24] A. Shiki, Y. Inoh, S. Yokawa, T. Furuno, Promotion of microtubule acetylation plays an important role in degranulation of antigen-activated mast cells, *Inflamm. Res.* 68 (2019) 181–184.
- [25] E.S. Place, N.D. Evans, M.M. Stevens, Complexity in biomaterials for tissue engineering, *Nat. Mater.* 8 (2009) 457–470.
- [26] R. Teshima, K. Suzuki, H. Ikebuchi, T. Terao, Possible involvement of phosphorylation of a 36,000-dalton protein of rat basophilic leukemia (RBL-2H3) cell membranes in serotonin release, *Mol. Immunol.* 23 (1986) 279–284.
- [27] T. Furuno, N. Hirashima, S. Onizawa, N. Sagiya, M. Nakanishi, Nuclear shuttling of mitogen-activated protein (MAP) kinase (extracellular signal-regulated kinase (ERK) 2) was dynamically controlled by MAP/ERK kinase after antigen stimulation in RBL-2H3 cells, *J. Immunol.* 166 (2001) 4416–4421.
- [28] R. Nakamura, T. Furuno, M. Nakanishi, The plasma membrane shuttling of CAPRI is related to regulation of mast cell activation, *Biochem. Biophys. Res. Commun.* 347 (2006) 363–368.
- [29] T. Furuno, N. Shinkai, Y. Inoh, M. Nakanishi, Impaired expression of the mitochondrial calcium uniporter suppresses mast cell degranulation, *Mol. Cell. Biochem.* 410 (2015) 215–221.
- [30] Y. Inoh, S. Tadokoro, H. Tanabe, M. Inoue, N. Hirashima, M. Nakanishi, T. Furuno, Inhibitory effects of a cationic liposome on allergic reaction mediated by mast cell activation, *Biochem. Pharmacol.* 86 (2013) 1731–1738.
- [31] Y. Inoh, A. Haneda, S. Tadokoro, S. Yokawa, T. Furuno, Cationic liposomes suppress intracellular calcium ion concentration increase via inhibition of PI3 kinase pathway in mast cells, *Biochim. Biophys. Acta* 1859 (2017) 2461–2466.
- [32] S. Yokawa, T. Furuno, T. Suzuki, Y. Inoh, R. Suzuki, N. Hirashima, Effect of cell adhesion molecule 1 expression on intracellular granule movement in pancreatic α cells, *Cell. Biochem. Biophys.* 74 (2016) 391–398.
- [33] A. Engler, L. Bacakova, C. Newman, A. Hategan, M. Griffin, D. Discher, Substrate compliance versus ligand density in cell on gel responses, *Biophys. J.* 86 (2004) 617–628.
- [34] Y.C. Yeh, J.Y. Ling, W.C. Chen, H.H. Lin, M.J. Tang, Mechanotransduction of matrix stiffness in regulation of focal adhesion size and number: reciprocal regulation of caveolin-1 and β_1 integrin, *Sci. Rep.* 7 (2017) 15008, <https://doi.org/10.1038/s41598-017-14932-6>.
- [35] Y. Horikoshi, T. Furuno, R. Teshima, J. Sawada, M. Nakanishi, Thapsigargin-induced nuclear calcium signals in rat basophilic leukaemia cells, *Biochem. J.* 304 (1994) 57–60.
- [36] M.M. Hamawy, S.E. Mergenhagen, R.P. Siraganian, Tyrosine phosphorylation of pp125^{FAK} by the aggregation of high affinity immunoglobulin E receptors requires cell adherence, *J. Biol. Chem.* 268 (1993) 6851–6854.
- [37] M.M. Hamawy, W.D. Swaim, K. Minoguchi, A.W. de Feijter, S.E. Mergenhagen, R.P. Siraganian, The aggregation of the high affinity IgE receptor induces tyrosine phosphorylation of paxillin, a focal adhesion protein, *J. Immunol.* 153 (1994) 4655–4662.
- [38] J.R. Pfeiffer, J.C. Seagrave, B.H. Davis, G.G. Deanin, J.M. Oliver, Membrane and cytoskeletal changes associated with IgE-mediated serotonin release from rat basophilic leukemia cells, *J. Cell Biol.* 101 (1985) 2145–2155.
- [39] J. Shan, Q. Chi, H. Wang, Q. Huang, L. Yang, G. Yu, X. Zou X, Mechanosensing of cells in 3D gel matrices based on natural and synthetic materials, *Cell Biol. Int.* 38 (2014) 1233–1243.
- [40] Y. Ho, S.H. Kok, J.S. Wang, L.D. Lin, Translucent titanium coating altered the composition of focal adhesions and promoted migration of osteoblast-like MG-63 cells on glass, *J. Biomed. Mater. Res.* A 102 (2014) 1187–1201.
- [41] A.J. Torres, L. Vasudevan, D. Holowka D, B. Baird, Focal adhesion proteins connect IgE receptors to the cytoskeleton as revealed by micropatterned ligand arrays, *Proc. Natl. Acad. Sci. U.S.A.* 105 (2008) 17238–17244.
- [42] N. O’Lunaigh, R. Pardo, A. Fensome, V. Allen-Baume, D. Jones, M.R. Holt, S. Cockcroft, Continual production of phosphatidic acid by phospholipase D is essential for antigen-stimulated membrane ruffling in cultured mast cells, *Mol. Biol. Cell* 13 (2002) 3730–3746.
- [43] A.F. Palazzo, C.H. Eng, D.D. Schlaepfer, E.E. Marcantonio, G.G. Gundersen, Localized stabilization of microtubules by integrin- and FAK-facilitated Rho signaling, *Science* 303 (2004) 836–839.
- [44] J.P. Dompierre, J.D. Godin, B.C. Charrin, F.P. Cordelières, S.J. King, S. Humbert, F. Saudou, Histone deacetylase 6 inhibition compensates for the transport deficit in Huntington’s disease by increasing tubulin acetylation, *J. Neurosci.* 27 (2007) 3571–3583.
- [45] F. Dubois, K. Alpha, C.E. Turner, Paxillin regulates cell polarization and anterograde vesicle trafficking during cell migration, *Mol. Biol. Cell* 28 (2017) 3815–3831.
- [46] S.P. Bhattacharyya, Y.A. Mekori, D. Hoh, R. Paolini, D.D. Metcalfe, P.J. Bianchine, Both adhesion to immobilized vitronectin and Fc ϵ RI cross-linking cause enhanced focal adhesion kinase phosphorylation in murine mast cells, *Immunology* 98 (1999) 357–362.
- [47] I. Kaverina, K. Rottner, J.V. Small, Targeting, capture, and stabilization of microtubules at early focal adhesions, *J. Cell Biol.* 142 (1998) 181–190.
- [48] E.J. Ezratty, M.A. Partridge, G.G. Gundersen, Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase, *Nat. Cell Biol.* 7 (2005) 581–590.
- [49] S.J. Stehbens, M. Paszek, H. Pemble, A. Ettinger, S. Gierke, T. Wittmann, CLASPs link focal-adhesion-associated microtubule capture to localized exocytosis and adhesion site turnover, *Nat. Cell Biol.* 16 (2014) 561–573.