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Integrin Signaling

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Abbreviations

ECM	Extracellular matrices
FACS	Fluorescence-activated Cell Sorting
FAK	Focal adhesion kinase
FAT	Focal-adhesion-targeting
FBS	Fetal Bovine Serum
FIH	Factor-inhibiting HIF
GEF	Guanine exhange factor
HIF-1	Hypoxia-inducible transcription factor 1
HRE	Hypoxic response element
MAPK	Mitogen activated protein kinase
MC	Mast cells
MC-CPA	Mast cell carboxypeptidase A
NDGA	Nor-dihydroguaiaretic acid
PBS	Phosphate buffered saline
PI	Propidium iodide
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinases
TM	Transmembrane
VEGF	Vascular endothelial growth factor

Summary

Integrins comprise a family of cell surface adhesion receptors responsible for interactions between cells and cells with extracellular matrix. As receptors without any kinase activity of their own, after activation and clustering they transfer signals across the plasma membrane, so called inside out and outside in signaling. Signaling pathways that involves integrins are the FAK/Src pathway, MAPK/Erk pathway, the PI3K/Akt pathway and others. Several important cellular functions such as anchorage dependent survival and cell proliferation are dependent on these interactions through generation of particular intracellular signals.

Integrin mediated regulation of the cell cycle is lost in most malignant tumor cells, resulting in anchorage independency of growth. In this project, the P19 EC (embryonal carcinoma) cell line and the Fucci HeLa cell line were used to study if they have the ability of growth independently of anchorage. Data show that both of them could pass through the G1 checkpoint but with some delay. However, better methods are needed in future work. Meanwhile, for blood cells it is not clear if they proliferate in circulation, independently of adhesion. Bone marrow mast cells were treated with EdU (5-ethynyl-2´-deoxyuridine), which is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis, in order to study their integrin independent proliferation rate. As a result, these non-adhesive cells are able to divide without integrin signaling, at least in this experiment.

Reactive oxygen species (ROS), toxic by-products of oxygen metabolism also has affect on integrin-mediated signaling. NGDA (Nor-dihydroguaiaretic acid) and Rotenone were used as inhibitors targeting different ROS production pathways; some effects on the phosphorylated state of key proteins in integrin signaling pathways were detected in this experiment. Vitamin C, an antioxidant that protects the cells from free radical damages, is also necessary for collagen secretion fibers. Fibroblast cells were cultured with Vitamin C in this experiment to study their effect on integrin signaling. However, no significant difference was observed between these culturing conditions and normal ones.

Introduction

1. Integrin structure and activation

Integrins are members of a large family of cell surface-adhesion receptors that are expressed in all metazoans. They are heterodimers of noncovalently associated α and β subunits, each of which is a single pass type I transmembrane protein. There are 18 different α chains and 8 different β chains in humans. They are composed of large extracellular domains (approximately 800 amino acid), transmembrane (TM) parts (about 20 to 30 amino acids) and short cytoplasmic tails (ranging in size between 13 and 70 residues, and form a total number of 24 heterodimers (Hynes 2002; Humphries, Byron et al. 2006). In general, integrins have three different conformations: an active (high affinity) state available for ligand binding where the extracellular parts are extended; an inactive (low affinity) state with a bent conformation of extracellular parts to which ligand cannot bind, and an intermediate state.

The major roles of integrins are to anchor cells to the extracellular matrices (ECM) and to change the function of cells through activating specific intracellular signaling pathways after ligand binding (called “outside-in” signaling). At the same time, they can shift between high- and low-affinity conformations for ligand binding generated by “inside-out” signaling; through these signaling pathways, the shift from a low- to a high-affinity state happens and is termed “integrin activation” (Figure 1). This property of integrin activation is initiated by signals generated from external cues via other receptors, that are transduced intracellularly and ultimately result in the binding of regulatory proteins such as talins and kindlins to integrin cytoplasmic domains.
(Hynes 2002; Legate and Fassler 2009).

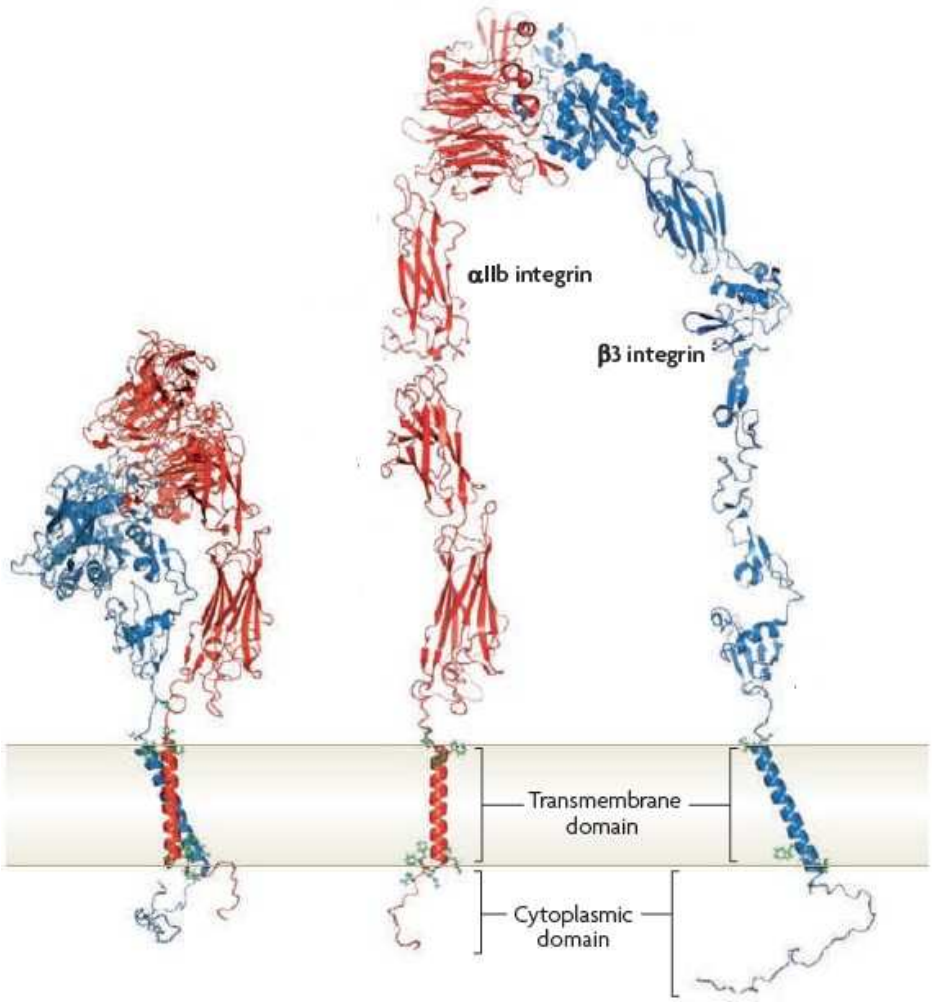


Figure 1. Integrin structures

Integrins are heterodimeric adhesive receptors consisting of an α and a β-subunit. The crystal structures are shown here as the 'bent conformation' (left) that can be unfolded to facilitate visualization of the domains (right). The transmembrane domain and cytoplasmic domain are shown inside the membrane (the grey area) The low affinity state of the integrin for its ligands is maintained by non-covalent interactions between the α- and β-integrin transmembrane and cytoplasmic domains (Lau et al. EMBO J 28 (2009), 1351-1361).

2. Integrin signaling pathways

The engagement of integrins during cell adhesion regulates gene expression and cell growth, differentiation, and survival (Schwartz, Schaller et al. 1995). These effects are achieved through signals generated by ligand-occupied and clustered integrins. As a receptor without any enzymatic activity on its own, the engagement of adaptors and signaling molecules as well as cell scaffolding structure is also required in the bidirectional signal transduction. Increased tyrosine phosphorylation of certain target

molecules and elevated lipid second messengers levels such as phosphatidylinositol-4,5-biphosphates (PtdIns-4,5-P2) and PtdIns-3,4,5-P3 occur upon integrin engagement. Even though integrins and F - actin do not bind directly to each other, they need to be physically connected to each other. Finally, partial depending on mechanical forces, cytoskeletal rearrangements take place, followed by subsequent changes in several pathways as well as in gene expression (Willard and Devreotes 2006).

The FAK/Src pathway

Focal adhesion kinase (FAK) was one of the first integrin signaling molecules that was identified as kinases specific to cell adhesion sites. As a phosphorylation-regulated signaling scaffold, it is important for adhesion signaling turnover, Rho-family GTPase activation, cell migration and cross-talk between growth-factor signaling and integrins (Mitra, Hanson et al. 2005). FAK is ubiquitously expressed in mammalian cells and is essential for life; knockout of the protein is embryonically lethal in mice. The protein contains a FERM domain at the N-terminal, a catalytic domain, a proline-rich regions and a C-terminal focal-adhesion-targeting (FAT) domain which interacts with talin as well as paxillin. During integrin signaling transduction and in response to integrin clustering, FAK autophosphorylation occurs and then generates docking sites for SH2 domain-containing proteins such as Src kinases. Src becomes activated upon binding to FAK and is in turn activated at the same time as phosphorylated FAK, resulting in enhancement of its kinase activity and interactions with other proteins. (Mitra and Schlaepfer 2006)

The MAPK/Erk pathway

Another major pathway that is triggered after integrin activation is the mitogen activated protein kinase (MAPK)/Erk pathway, which is also activated by ligand binding to receptor tyrosine kinases (RTK), for instance the EGF and PDGF receptors. A simplified description of this pathway by growth factors is that binding of ligand to extracellular domains induces dimerization of the receptors and consequent autophosphorylation of tyrosine residues in the cytoplasmic domains. Binding of the Grb2/SOS complex to specific phosphotyrosines leads to activation of Ras by SOS (a guanine exchange factor, GEF). GTP-loaded Ras binds to and activates Raf kinase, which phosphorylates and activates Mek 1 and 2. Phosphorylated Mek 1 and 2 activate Erk 1 and 2. Activated Erk then phosphorylates target proteins both in the cytosol and in the nucleus (Scaltriti and Baselga 2006; McKay and Morrison 2007).

3. Reactive oxygen species (ROS)

Oxygen is an essential factor in a great number of biological events of aerobic

organisms. Tissue and cellular regulation of oxygen supply is vital to mediate adaptation mechanisms during low or high oxygen conditions.

Reactive oxygen species (ROS), including superoxide anions ($O_2^{-\cdot}$) and hydrogen peroxide (H_2O_2), known toxic by-products of oxygen metabolism, fulfill various tasks in the immune response. However, it is currently recognized that being produced at lower and regulated levels, ROS can act as second messenger involved in the transduction of mitogenic, differentiation, and adhesion signals (Finkel 1998; Finkel 2000; Finkel 2003). At present, the mechanisms for ROS production and its molecular targets remain unclear. However, there are three distinct mechanisms identified. During neutrophil activation, superoxide is produced by a membrane oxidase (NOX2) in the phagosome to kill the endocytosed pathogen. In non-phagocytic cells, ROS is produced by a distinct members of the NOX family oxidase at the plasma membrane, inhibits phosphatases and elevates tyrosine kinases signaling (Lee, Kwon et al. 1998; Barrett, DeGnore et al. 1999). ROS associates with receptor activation for example during stimulation of EGF (Bae, Kang et al. 1997) and PDGF receptors (Sundaresan, Yu et al. 1995). The third and the most important source for ROS production in all cell types is the mitochondria where 1–5% of the transported electrons is diverted into formation of superoxide instead of water (Boveris, Oshino et al. 1972). However, due to the short-lived nature of ROS and the unspecific effect of inhibitors, it is not well known how does mitochondria switch to ROS production involves in modification of its function, because multiple pathways may have effect on this process. Furthermore, recent studies reveal that the production of ROS may be accompanied by changes in mitochondrial metabolism (Nemoto, Takeda et al. 2000; Nicholls and Budd 2000). The producing rate of mitochondrial superoxide is regulated during TNF signaling (Kunz, Kuznetsov et al. 1993; Sanchez, Zhang et al. 2000), hypoxia (Vanden Hoek, Shao et al. 1997; Chandel, Maltepe et al. 1998), and apoptosis (Cai and Jones 1998), where the small GTPase Rac has emerged as a common mediator. (Irani, Xia et al. 1997; Page, Li et al. 1999; Ozaki, Deshpande et al. 2000; Suzukawa, Miura et al. 2000).

GTP-Rac appears to be involved also in the activation of other ROS-generating enzymes. Mutations in Rac are used to identify its interactions with effector proteins during molecular mechanisms. For example, the best understood mechanism is elevated ROS production in neutrophil, Rac2 binds to a NOX (NAD(P)H oxidase) to mediate assembly and function of the enzyme complex burst oxidase at the plasma membrane (Freeman, Abo et al. 1996). Werner and Werb revealed that the transient activation of the small GTPase Rac, followed by a rise in reactive oxygen species (ROS), are necessary early steps in a signal transduction cascade that lead to NFκB activation and collagenase-1 (CL-1)/matrix metalloproteinase-1) production after integrin-mediated cell shape changes (Werner and Werb 2002). Chiarugi and coworkers outlined a redox circuitry upon cell adhesion by using different inhibitors; oxidative inhibition of a protein tyrosine phosphatase was found to promote the phosphorylation activation and the downstream signaling of FAK (Taddei, Parri et al.

2007). As a final event according to their data, fibroblast cells adhere and spread on fibronectin. Taddei et al. (2007) reported that in murine NIH3T3 fibroblasts, a mitochondrial ROS release occurs, strictly confined to the early phase of extracellular matrix (ECM) contact. Besides, 5-lipoxygenase (5-LOX) is reported to be engaged by integrin receptor ligation as another ROS source, contributing to the more-intense, second ROS burst, possibly orchestrating the spreading of cells in response to ECM contact (Taddei, Parri et al. 2007).

Under low oxygen conditions, hypoxia-inducible transcription factor 1 (HIF-1) is a key regulator of responses, which specifically binds to hypoxic response element (HRE) in DNA and thereby activates the transcription of a number of genes including vascular endothelial growth factor (VEGF), heme oxygenase, glucose transporter-1, and erythropoietin (Semenza, Nejfelt et al. 1991; Semenza and Wang 1992; Lee, Jiang et al. 1997). As a heterodimeric complex, HIF-1 is composed of HIF-1α and HIF-1β, whereas HIF-1α is the oxygen-regulated subunit (Wang, Jiang et al. 1995). Regulation of HIF-1α occurs in two ways: proline hydroxylation targets the protein to the proteasome for degradation by binding to pVHL (von Hippel-Lindau protein) so that under normoxic conditions HIF-1α is undetectable (Mole, Maxwell et al. 2001; Hirsila, Koivunen et al. 2003; Schofield and Ratcliffe 2004); on the contrary, asparagine hydroxylation by factor-inhibiting HIF (FIH) prevents assembly of a viable transcriptional complex (Lando, Peet et al. 2002; Lee, Kim et al. 2003; Masson and Ratcliffe 2003; Linke, Stojkoski et al. 2004). Recent studies have shown that hydroxylation on proline 402 is altered by Ang II, decreasing pVHL binding to HIF-1α and allowing HIF-1α protein to escape subsequent ubiquitination and degradation mechanisms. HIF-1α stability is mediated through the Ang II–mediated generation of hydrogen peroxide and a subsequent decrease in ascorbate levels, leading to decreased HIF prolyl-hydroxylase activity and HIF-1α stabilization (Vissers, Gunningham et al. 2007).

Hypoxia inducible factor (HIF)-1 is modulated by hydroxylation of the proline and asparagine hydroxylases that control transcription factor HIF-1 require ascorbate for optimal activity. Vissers and coworkers investigated the effect of intracellular ascorbate on HIF-1α protein levels and HIF-1-mediated gene expression in two human primary cell lines (umbilical vein endothelial cells and skin fibroblasts) together with one human cancer cell line (A431 epithelial cells), and their results proved that intracellular ascorbate is a major regulator of the hypoxic responses in normal cells and optimal levels of this vitamin will have a profound effect on HIF-1-regulated processes. (Vissers, Gunningham et al. 2007)

Vitamin C, a well-known antioxidant that protects cells from free radical damages, is also necessary to form collagen, an important protein used to make skin, scar tissue, tendons, ligaments, and blood vessels. During in *vitro* experiments, cells are commonly kept devoid of vitamin C, which may lead to erroneous results due to cellular stresses. In particular, the involvement of vitamin C in the production of nitric

oxide (NO) has attracted much attention. Ascorbate has been shown to specifically protect tetrahydrobiopterin (BH4), cofactor of the endothelial nitric oxide synthase against oxidation that is crucial for the proper function of eNOS (Heller, Munscher-Paulig et al. 1999; Heller, Unbehaun et al. 2001). Impaired bioavailability of NO results in endothelial dysfunction; thus, ascorbate may participate in the prevention of this condition (Pacher, Beckman et al. 2007). A major default in cell culture experiments with vitamin C is its rapid deterioration of ascorbate in the medium, causing the vitamin to irreversibly degrade within hours (Chepda, Cadau et al. 2001). Using combinations of ascorbate and 2-phosphoascorbate, Frikke-Schmidt, and Lykkesfeldt (2009) found that a single supplement of ascorbate and 2-phosphoascorbate adequately maintains intracellular vitamin C at physiological levels for up to 72 hours. Our group is interested in the vitamin C effect in fibroblast cells and the possible influence of integrin-induced ROS production on vitamin C levels.

4. Anchorage independent growth

The cell cycle is a complicated process required for cell proliferation, organismal development, tissue hyperplasia (as a response to injury), and diseases such as cancer, which is generated by deregulated cell cycle. The cell cycle can be morphologically subdivided into interphase, which consists of G1, S, G2 phases, stages of M (mitotic) phase which include prophase, metaphase, anaphase and telophase, and cytokinesis (Schafer 1998). For many adherent cell types, a reduction in cell contacts accompanied by cell rounding occurs during entry to mitosis. The subsequent cytokinesis and re-entry to G1 both require cell attachment, as well as the involvement of focal adhesion re-establishment. Commensurate with such a dynamic attachment profile, many cell adhering proteins such as integrins are post-translationally modified and/or re-localized during mitosis (Doxsey, McCollum et al. 2005). However, loss of cell adhesion is one of the events that tumor cell achieves during tumorigenesis.

The requirement for integrin mediated cell adhesion in G1-phase progression of normal cells has already been well characterized (Pugacheva, Roegiers et al. 2006). Adhesion-dependent signaling mediated by integrins cooperates with mitogenic signaling to phosphorylate the retinoblastoma protein, Rb, thereby releasing E2F, the function of which is required for G1 to S phase progression. In absence of this signaling, Rb remains hypo-phosphorylated and E2F is not released, therefore resulting in cell cycle arrest when cells are kept in suspension. However, in SV40 LTAg transformed cells, SV40 LTAg interacts with Rb as well as with p53 (Li, Zhao et al. 2003). SV40 LTAg-mediated inactivation of Rb and p53 overrides G1 arrest. In contrast, the molecular feature of adhesion signals required for cytokinesis is not well understood. Recent studies have shown that integrin trafficking to the cleavage furrow is a necessary part of the mechanism (Pellinen, Tuomi et al. 2008). Oncogenic Ras mutants (constitutively active) overcome the cytokinesis block of cells in suspension, suggesting the importance of cytokinesis block in preventing tumorigenesis

(Thullberg, Gad et al. 2007). Furthermore, keeping adhesion-dependent cells in suspension culture has been shown to result in the cell cycle being arrested in cytokinesis, which results in bi- or multi-nucleation of cells (Ben-Ze'ev and Raz 1981; Thullberg, Gad et al. 2007).

5. Mast cell

Mast cells (MC) are an important part of our immune system. When re-exposed to an allergen or a trigger that our body senses as a foreign invader, mast cells release chemicals including histamine that quickly travels through the body to fight off these antigens. MCs originate from bone marrow precursors expressing the CD34 molecule, which then circulate in the blood as immature precursors, and then migrate into various tissues where they undergo terminal differentiation under influence of local growth factors, in particular stem cell factor (SCF) (Gurish and Boyce 2002). In mature MCs, there are numerous electron dense granules in the cytoplasm that can be released following an appropriate stimulus. In the degranulation process, they release a number of preformed components to the exterior (Metcalfe, Baram et al. 1997; Galli, Kalesnikoff et al. 2005; Galli, Nakae et al. 2005). MC granules contain proteoglycans, cytokines such as TNF-α (Gordon, Burd et al. 1990), and a number of MC-specific proteases. Tryptases and chymases belong to the serine protease class and MC carboxypeptidase A (MC-CPA) of zinc-dependent metalloproteases, respectively. These proteases are very efficient cell markers for distinguishing different stages and types of MCs.

In culture, in contrast to adhered cells, most of the bone marrow MC cells are floating as cell clusters while some cells adhere to the dish. The doubling time of cell number in these cultures is 8-10 days, suggesting that the proliferation is limited to a small subpopulation of cells. Our group is interested in clarifying whether MC can grow anchorage–independently or if they require integrin signals during cell cycle progression.

Results

Colony formation of P19 cells

The soft agar assay for colony formation is a well-established method to study anchorage-independent growth, and thus for detecting malignant transformation of cells. Normal (non-transformed) adherent cells can only divide when they are attached to a solid surface. When suspended in a viscous fluid or a gel, for example agar in this soft agar assay experiment or agarose, they fail to grow and instead undergo apoptosis. In the experiment, in order to determine the colony formation of different adherent cell lines, the stem cell line P19 EC (an embryonal carcinoma-derived cell line from mouse female embryos) and positive control MCF 7 (a breast cancer cell line) were analyzed. The MCF 7 cell line could form colonies in the assay which is the feature of cancer cell line, and this served as a positive control for this experiment. P19 cells were found to be able to form colonies.

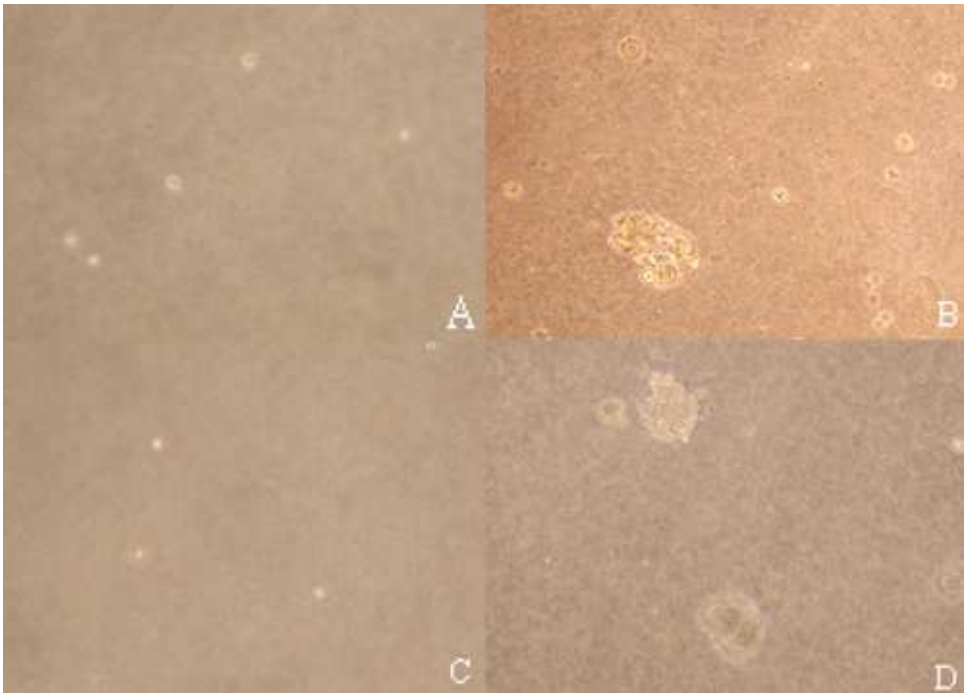


Figure 2. Colony formation of P19 cells and MCF 7 cells.
P19 cells and MCF 7 cells were seeded at a density of 1000 cells per well in 6 well plate, with 10 % Fetal bovine serum (FBS) in 1% mg/ml soft agar. Pictures were taken under 100 x magnification. (A) P19 cells at day one after seeding. (B) P19 cells at day ten after seeding. (C) MCF 7 cells at day one after seeding. (D) MCF 7 cells at day fourteen after seeding.

Cell cycle analysis of P19 cells and Fucci-HeLa cells by FACS analysis

Flow cytometry (FACS) is a technique for measuring certain physical and chemical characteristics of cells or particles by suspending them in a stream of fluid and passing them one at a time by an electronic detecting apparatus. The voltage pulse

converted through this process could be measured in three values: height (H), area (A), and width (W) of the pulse. To investigate the distribution of cells in different cell cycle stages, the DNA content of collected M phase cells and suspension cells, both P19 and Fucci HeLa cells, was analyzed (Figure 3). Control cells (P19 cells and Fucci HeLa cells) were collected right after trypsin harvest. M phase (mitotic phase) cells were collected by both mitotic shake off (P19 cells; Fucci HeLa cells) and incubation with nocodazole M phase synchronization reagent for 3 hours (P19 cells). All P19 samples were fixed with 70% methanol and stained with propidium iodide of their DNA, while the Fucci HeLa cells were stained with Hoechst 33342 after fixation in 70% ethanol. Only P19 cells (A) were able to show the cell cycle distribution: G1: 44.7%, G2: 19.8%, G2/M: 35.3%. The other samples gave results that we could not interpret.

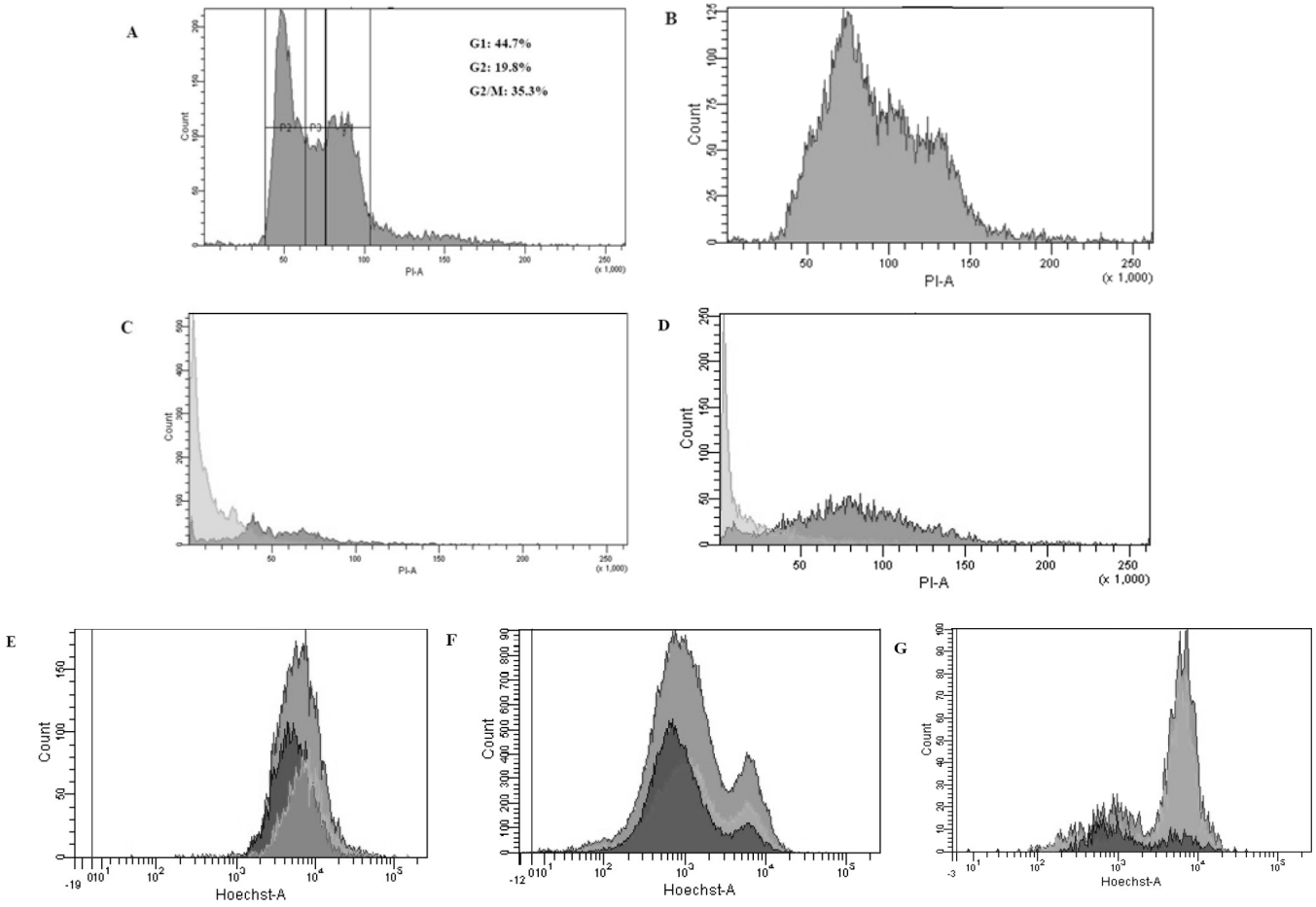
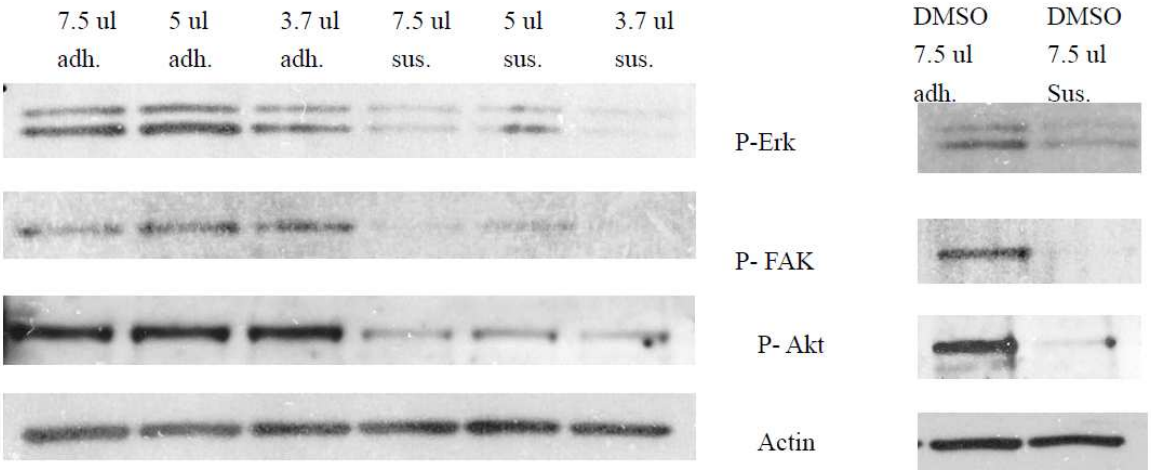


Figure 3. Cell cycle distribution and M phase cell quality of P19 cells and Fucci HeLa cells as measured by flow cytometry. Each peak represents different DNA content of cells. (A): Histogram representation of the cell cycle distribution of exponentially growing P19 cells. (B): Histogram representation of the cell cycle distribution of exponentially growing P19 cells of abnormal quality. (C): Histogram representation of the P19 M phase cell collected by mitotic shake off. (D): Histogram representation of the collected P19 M phase cell incubated with Nocodazole. (E): FACS analysis of the cell cycle distribution of exponentially growing Fucci HeLa cells. (F): Representation of the Fucci HeLa M phase cell collected by mitotic shake off. (G): Representation of the collected Fucci HeLa M phase cell kept in suspension for 5 hours.

Integrin signaling

Rotenon and Nor-dihydroguaiaretic acid (2, 3-dimethyl-1, 4-di [3, 4-dihydroxyphenyl] butane, NDGA) are potent inhibitors of the mitochondrial electron transport systems and lipoxygenase, respectively. Vitamin C is an antioxidant that protects the cells from free radical damage, as well as a necessary cofactor for enzymes involved in collagen secretion and HIF-1 stability. To determine the regulatory effects of Rotenone, NDGA and Vitamin C on integrin signaling, phosphorylation of Erk, Akt and FAK were analyzed in GD25 β1 a cells. Cell adhesion assay was preformed, by treating cells with different Rotenon and NDGA with different concentrations for 15 minutes, followed by western blotting after harvested the cells. These results show different inhibitors had variable influence on respective signaling kinases.

A :



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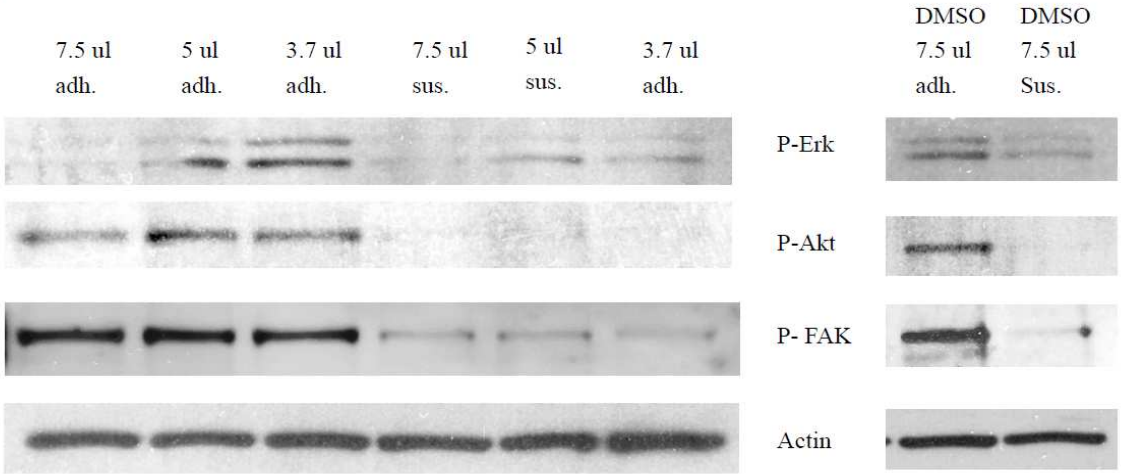

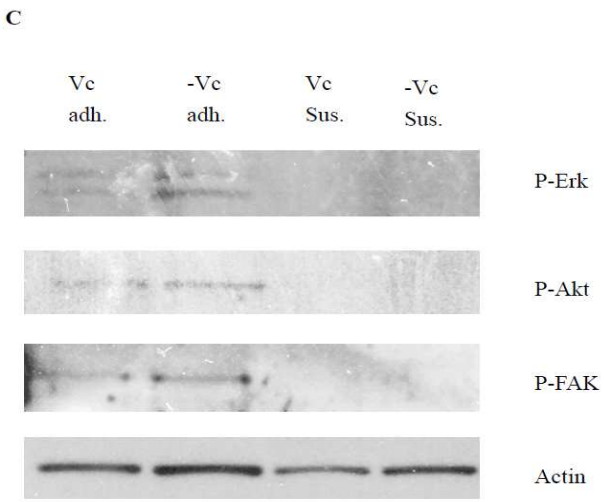

Figure 5. Effect of Rotenone, NDGA and Vitamin C in integrin signaling
GD25β1A cells were kept in suspension or seeded on invasion-treated dishes for 60 min. Rotenone (A) and NDGA (B) was preincubated with the cells for 15 minutes before the experiment. After cell lysis, the phosphorylation of Erk, Akt, and FAK was detected by western blot. Actin is shown as loading control. (C) GD25β1 A cells grown in the presence or absence of vitamin C for 7 days were analyzed in the cell adhesin assay as described above.

Mast cell proliferation in suspension

To measure the proliferation ability of 4 weeks old bone marrow mast cells in suspension culture, the cells were incubated with the EdU (5-ethynyl-2´-deoxyuridine) for 24 hours in a concentration of 2.5 μm, whereby DNA synthesis in proliferating cells is detected, based on the incorporation of the fluorescent nucleotide analogue (Figure 4). Cytospin is preformed after harvesting of the cells. Nuclei were stained with DAPI (blue). Different cytoplasm granule markers (FITC green) were used to determine the maturation stage and purification of mast cells: MC carboxypeptidase A (MC-CPA, Figure 4 B) is a zinc-dependent metalloprotease, chymase (MMCP-4, Figure 4 C) belong to the serine protease class, and while tryptase (MMCP-6, Figure 4 D).

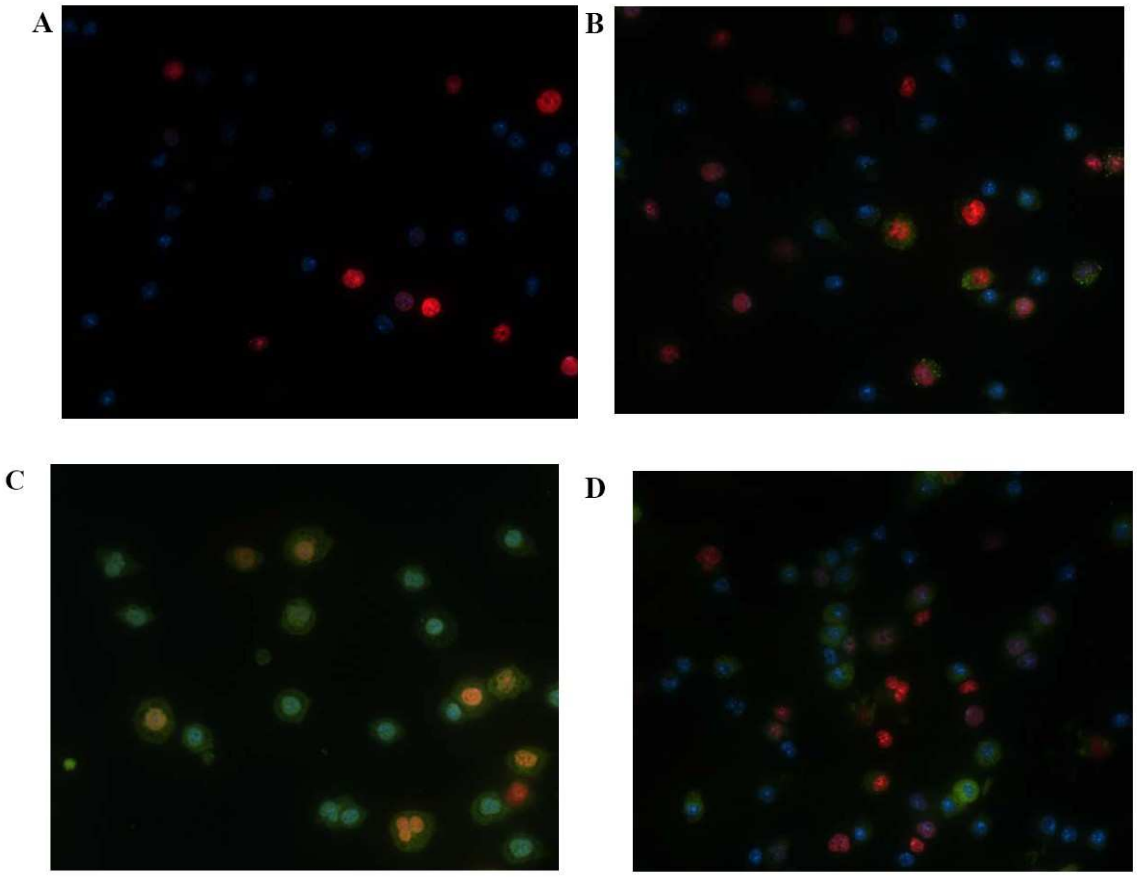

Figure 4. Bone marrow mast cells progress in proliferation

All cells were incubated with 2.5 μM EdU for 24 hours before harvest (stains the nuclei of proliferating cells cyc 3, nuclei of normal cells DAPI, granules with different tryptase or chymase FITC). (A) Bone marrow mast cells stained with only secondary Ab (negative control). (B) Bone marrow mast cells stained for alpha-CPA. (C) Bone marrow mast cells stained for MMCP-4. (D) Bone marrow mast cells stained for MMCP-6. Pictures were taken at 40 x magnification.

Bone marrow mast cell proliferation rate

To analyze the proliferation percentage of bone marrow mast cells, a total number of around 340 to 430 cells were counted and different rate of immunofluorecent staining positive cells were also calculated in order to show more detailed information. EdU positive cells were the ones that proliferate, DAPI were used to stain the nucleus, cells were treated with three kinds of cytoplasm granule markers: α - CPA, MMCP-4, MMCP-6. After staining, cells were counted: total number of cells (nucleus stained with DAPI); number of mast cell granule marker positive cells (granules stained with FITC); number of proliferation positive cells (nucleus stained with EdU Cyc 3). 60 % cell viability was observed. The result is given as numbers of positive cells / total number of cells.

IF staining marker	DAPI	Mast cell marker	EdU	DAPI	Mast cell marker	EdU	DAPI	Mast cell marker	EdU	DAPI	Mast cell marker	EdU
Usage of the marker	+	-	-	+	+	-	+	-	+	+	+	+
Calculated groups	Total number of cell			Mast cell granule marker positive cells			Proliferation positive cells			Proliferation and mast cell granule marker positive cells		
2^{nd} Ab control	341			-			31.1%			-		
α - CPA	395			80.3%			30.1%			21.1%		
MMCP-4	335			78.5%			29.0%			18.8%		
MMCP-6	422			63.7%			28.2%			12.6%		

Viability: 60%

Table 1.

Calculated results from bone marrow mast cell EdU staining data with 60 % viability. In the content, 2^{nd} Ab control staining, there were 341 cells counted, whereas 31.1% were in proliferation. There were 395 Alpha CPA stained cells counted, 80.3% of the cells were in Alpha CPA positive cells, 30.1% cells are in proliferation, 21.1% of them were in the proliferation process. 335 MMCP-4 stained cells were counted, 78.5% of the cells were in granule marker MMCP-4 positive cells, 28.9% cells are in proliferation, 18.8% of them were in the proliferation process. 422 MMCP-6 stained cells were counted, 63.7% of the cells were in MMCP-6 granule marker positive cells, 28.2% cells are in proliferation, 12.6% of them were in the proliferation process.

Discussion

Anchorage independent growth

The aim of this part of the experiment was to study if the stem cells like cells could perform cytokinesis in a anchorage independent manner. Two different adherent cell lines MCF 7 (a breast cancer cell line), P19 EC (an embryonal carcinoma-derived cell line from female embryos) were studied. Therefore time-lapse video were preformed as an initial study. After that, cells were grown in soft agar for colony formation measurement. Further, their cell cycle distribution was analyzed by cell flow cytometry. In soft agar assay, MCF7 cells as the positive control could form colony after 14 days. P19 cells also formed colonies that were clearly detectable 10 days after seeding. However, time-lapse video-microscopy indicates that mitotic phase (M phase) P19 cells in suspension culture could not pass the mitotic checkpoint within 8 hours (not shown in this report). Further, combining the results in FACS analysis shows that the cell cycle distribution of P 19 cells was G 1 phase around 5-6 hours, G2 phase around 2-3 hours, M phase around 4-5 hours. Hereby, in this experiment, P 19 cells were able to pass through the cytokinesis block and carry on their proliferation in an anchorage independent manner. This delay is likely to be due to the lack of integrin-mediated signals, which is an essential condition for untransformed normal adherent cell lines. Instead of having delayed M phase, they undergo apoptosis. In the mean time, the colony formation characteristic of P 19 cells in soft agar assay also indicated that P19 cells have the feature of anchorage independent growth similar to cancer cell lines such as Fucci-HeLa cell line (a fluorescent protein expressing HeLa cell line). On the other hand, in FACS analysis results, compared to normal adherent cells, P19 cells display relatively short G1 phase, which is a typical feature of embryonic stem cells.

However, there are aspects that could be improved in later experiments. The culturing method was not optimal in this experiment and the cell cycle distribution was not clearly separated. Possibly, the use of other stem cell media or a reduced serum concentration could improve the culture conditions. M phase cells were collected by mitotic shake off, but unfortunately most of them were dead cell particles or apoptotic cells here. In order to improve the quality of M cell, nocodazole (a chemical that interferes with polymerization of microtubules in the cell) was incubated with P19 cells for 4 hours before collection. However, cells were not compatible with the method protocol we used, and as a result the quality of the cells still showed no big improvement. Being a stem cell like cell line, it is possible that P19 is more sensitivity when treated with different reagents than other cell lines.

As for another interesting cell line, Fucci HeLa cells, the time-lapse video-microscopy confirms that they could pass though the G1 check point and progress to the next cell cycle (data not shown), which is typical for carcinoma cell lines. When analyzed by

FACS, by comparing the differences between adherent control and anchorage independent growth condition (5 hours in suspension), G2/M phase cells were accumulated after suspension, which appeared to contradict our previous results that as a carcinoma cell line Fucci HeLa cells were able to pass through cytokinesis independent of anchorage signaling without much delay. Possibly, this might be due to the variation of suspension culture. Improved suspension culture methods, such as inclusion of methyl cellulose in the culture medium to reduce diffusion of the cells could be used in further work to determine a reasonable suspension time point.

Integrin signaling

The aim of this part of the experiment is to study the influence of ROS produced by mitochondria and lipoxygenases (LOX) as well as vitamin C in key proteins in integrin signaling such as phosphorylated Akt, Erk and FAK. During the experiment, GD 25 β1 a cells (integrin β1 knockout mouse fibroblast cell line) were starved with serum free DMEM media for 24 hours, let spread and adhere for one hour, then during the final 15 minutes treated with different concentrations of NDGA and Rotenone. According to literature, NDGA (nordihydroguaiaretic acid) is an inhibitor of cell cytosolic oxidases that block the ROS production of these enzymes and inhibit or delay the cell adhesion and spreading. The western blotting date showed there was relatively little influence between each concentration on phosphorylated Akt, FAK and Erk in suspension cells, as well as phosphorylated Akt and FAK in adhesion cells. Also little differences were shown between respective controls and those conditions. It appears that NDGA influenced adherent cells where integrin-mediated signaling takes place. As for phosphorylation of Erk, the more NDGA was added the better inhibition effect it had. Rotenone as another reagent in this experiment interferes with the electron transport chain in mitochondria. It inhibits the electron transfer from iron-sulfur centers in complex I to ubiquinone. This prevents NADH from being converted into cellular energy (ATP). In this experiment, however, its influence was rather small on the investigated phosphorylation reactions. The antioxidant vitamin C is also important for collagen formation, which makes its influence on cell culture, especially on fibroblast cell lines valuable to study. Adding Vitamin C in the cell culture media and constantly growing GD 25 β1 a cells in such condition, followed by cell adhesion assay and western blotting, did not result in any obvious enhancement of phosphorylation of the key kinases in integrin signaling compared with control in both adhesion and suspension conditions.

Bone marrow mast cell proliferation

The aim of this part of the experiment was to clarify whether the proliferation of the non-adherent mast cells is dependent on integrin involvement or not. EdU (5-ethynyl-2´-deoxyuridine), a chemical which incorporate with DNA during S phase in dividing cells, was used to incubate mast cells for their proliferation detection.

Different granules in mast cells that distinguish various stages of their maturation were detected by respective granule markers. Around 30% of the cells proliferated as determined by EdU-staing. Granule stainings showed that alpha-CPA, MMCP-4 and MMCP-6 positive cells were proliferating. By comparing with the control, there is not much unspecific cytoplasm staining. From these data, we could come to the conclusion that a significant fraction of the mast cells in this experiment did proliferate in suspension culture，and that the more mature they were the less they proliferate. However, as an initial experiment, there were aspects that could be improved. The cells were grown in a normal cell culture dish, so that it could not be entirely excluded that the cells transiently attached to the dish, producing the integrin signal for cell division. Moreover, mast cell could form cell aggregates in the culture media, and these cell-cell contacts may also provide this kind of integrin signal. In future work, we can use a special culture dish to prevent cell adhesion to the dish and use methyl cellulose to avoid formation of cell aggregates.

Materials and Methods:

Cell culture:

The following cell lines were maintained in DMEM supplemented with 10 % serum and antibiotics (50 μg/ml Penicillin and 60 μg/ml Streptomycin complete medium) at 37°C in a humidified 5% CO2 containing incubator (Velling, 2008): GD25β1A (integrin β1-deficient mouse fibroblast with transfected β1 A subunit) (Fassler, 1995) and Fucci HeLa cells (Sakaue-Sawano, 2008). Synchronized M-phase cells were obtained by the shake-off method using flasks containing exponentially growing cells. Suspension culture of cells was performed in dishes coated with 1% pluronic which prevent the cell and surface interaction, different cell suspension time points were set when performing the experiment.

Time lapse movie

Both suspension GD 25 β1 and P 19 cells (suspended in methylcellulose or media) and adhesion and adhesion cells for control experiment were grown in 35 mm petri dishes. Then, placed in the motorized stage of an Axiovert 200M-inverted microscope (Carl Zeiss) equipped with a cell culture chamber with constant supply of humidified 5% CO2 and temperature control at 37 ℃.

Soft-agar assay:

Soft agar assay was preformed in 6 wells plate. A bottom layer of 1 ml, 0.8% agarose in complete medium was made. On top, a layer containing 1000 P19 cells mixed in 1 ml 0.35% agar in complete medium was spread. Cells were incubated at 37°C in a humidified 5% CO2 containing incubator either for 10 days (P19 cells) or 14 days (MCF7 cells). The cell colony formation was checked every day to see the differences in growth. Photographs were taken in a dissecting microscope.

FACS analysis

GD 25 β1 Cells and P 19 cells were trypsinized and collected, washed twice with PBS, fixed by 70% methanol and then stained with propidium iodide (PI) containing 50 μg/ml PI, 20 μg/ml RNaseA, 0.1% sodium citrate and 0.1% triton X-100 in PBS, for 30 min in 37 ℃. In order to avoid cell aggregates, samples were passed through a 50 μm filter. Samples were analysis by BD FACSDiva software.

Cell adhesion assay and western blotting

To study the effect NDGA (inhibitor selective for lipoxygenase) and Vitamin C on integrin-induced signals in GD25β1A cell line, a cell adhesion assay (15 A) was preformed. Cell culture 6 well plates were coated with 10 μg/ml GST-invasin (for cells adhere to the surface) or pluronic (for cell inhibit of adhesion of surface). GD25β1A cells were serum-starved for 24 hours and then detached with trypsin-EDTA. Trypsin was inactivated with soybean trypsin inhibitor (1 mg/ml), and the cells were washed once in serumfree DMEM, and left to rest in suspension in serum-free DMEM at room temperature for 1 hour in order to down-regulate any remaining integrin signals. During the last 15 minutes of the suspension period, NDGA was added to the culture. The cells were subsequently seeded to previously coated culture plates for 1 hour at 37℃.

In order to analysis phosphoproteins by western blotting, the cells were lysed in SDS sample buffer containing 40 mM DTT, aspirated through 27G injection needle to shear the DNA, then heated at 95℃ for 3 minutes. The samples were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell; Germany). The membranes were blocked in 5 % milk in TBS-tween and incubated with the respectively phosphoprotein antibodies. For loading control, the membranes were probed with an anti-actin antibody Actin HRF, a goat polyclonol IgG (Santa Cruz Biotechnology). For detection of different phosphoralated proteins, primary antibody for pErk phosphor-p44/42 MAPK (Thr 202/Tyr 204) E 10 monoclonal was used (Cell Signaling Technology); for phosphorylated FAK rabbit primary antibody (PY ^{397}) was used (Invitrogen California). Reactive bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

Immunofluorescence staining

EdU (5-ethynyl-2´-deoxyuridine) detection was done according to the manufacturer instruction. Bone marrow mast cells were incubated with 2.5 μm EdU for 24 hours, followed by cytocentrifugation. Cells were fixed with 4% formaldehyde for 10 min, washed three times with TBS. Then stained by EdU staining mixture containing 100mM Tris, 0.5-1 mM $CuSO_4$, 1-100 μ M fluorescent azide and 50-100 mM ascorbic acid, followed by washing with TBS with 0.5% Triton X–100. Nuclei were conterstained with Hoechst. The cytoplasmid granules were stained with rabbit anti-granule marker alpha-CPA, MMCP-4 and MMCP-6 polyclonal antibodies (made by Magnus Åbrink's group).

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