THE ROLE OF RGD-ROSETTE NANOTUBES IN MIGRATION AND APOPTOSIS OF BOVINE NEUTROPHILS

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By

Le Minh Hong Anh

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ABSTRACT

Bovine respiratory disease complex is the most common disease that causes significant economic loss, typically in feedlot cattle. Current treatment methods are focused on reducing inflammatory responses, control of airway reactivity and improvement of pulmonary functions without potential side effects. Neutrophils are the key contributors in acute lung inflammation. However, activated neutrophils live longer and cause excessive tissue damage upon migration into lungs. Therefore, modulation of their migration and lifespan are attractive approaches in treatment strategies of bovine respiratory disease. Nanotechnology holds significant potential to design new compounds by our ability to manipulate at the nanoscale. Helical rosette nanotubes are a class of novel, biologically inspired, water soluble and metal-free nanotubes. I used helical rosette nanotubes conjugated to arginine-glycine-aspartic acid (RGD-RNT) to study their effects on neutrophil chemotaxis, cell signaling and apoptosis. Bovine neutrophils exposed to 5% RGD-RNT reduced their migration in response to fMLP (formyl-Methionyl-Leucyl-Phenylalanine), compared to the non-treated group ($P<0.001$). This inhibitory effect was the same as that of groups treated with ERK1/2 inhibitor (UO126) and p38 MAPK inhibitor (SB239063). In addition, the phosphorylated ERK1/2 and p38 MAPK for the first time were quantified by sandwich ELISA to elucidate the mechanism of neutrophil migration. The phosphorylation of both the ERK1/2 and p38 was inhibited at 5 minutes by RGD-rosette nanotubes ($P<0.05$). Furthermore, integrin $\alpha v\beta 3$ is possibly involved in migration of bovine neutrophils. Moreover, RGD-RNT did not induce apoptosis of bovine neutrophils which was inversed by pre-exposing them to LPS for 30 minutes ($P<0.001$).

These experiments provide the first evidence that RGD-rosette nanotubes suppress phosphorylation of ERK1/2 and p38 MAPK and inhibit chemotaxis of bovine neutrophils.

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LIST OF ABBREVIATION

BRD:	Bovine Respiratory Disease
BVD:	Bovine Viral Diarrhea
BRSV:	Bovine Respiratory Syncytial Virus
CNT:	Carbon Nanotubes
ECM:	Extracellular Matrix
ERK:	Extracellular Signal Regulated Protein Kinase
FAK:	Focal Adhesion Kinase
fMLP:	formyl-Methionyl-Leucyl-Phenylalanine
HSP 27:	Heat Shock Protein 27
IBR:	Infectious Bovine Rhinotracheitis
ICAM-1:	Intercellular Adhesion Molecule
IL-1:	Interleukin-1
LPS:	Lipopolysaccharide
MAPK:	Mitogen-Activated Protein Kinase
MLCK:	Myosin Light Chain Kinase
MWCT:	Multi-walled Carbon Nanaotubes
NOS:	Nitric Oxide Synthase
PMN:	Polymorphonuclear Leukocytes
PI-3:	Parainfluenza-3 virus
RGD-RNT:	RGD-Rosette Nanotubes
ROCK:	Rho Kinase

ROS:	Reactive Oxygen Species
RNS:	Reactive Nitrogen Species
RTK:	Receptor Tyrosine Kinase
SWNT:	Single-walled Carbon Nanotubes

1. INTRODUCTION

1.1. Bovine respiratory disease

Bovine respiratory disease complex (BRD) is the most common disease that causes significant economic loss, typically in feedlot cattle [1]. It is documented that the incidence of BRD in feedlot cattle varies from 4.6 to 43.8% in the United States from 1987 to 2001. Importantly, the number of calves observed with BRD drastically increases during the early days of their entry into the feedlot. The BRD complex accounts for lower average daily gain, increases in cost of treatment, mortality and culling rate [2].

Bovine respiratory disease is caused by multiple pathogens including viruses (bovine respiratory syncytial virus, BRSV; infectious bovine rhinotracheitis, IBR; parainfluenza-3 virus, PI-3; bovine viral diarrhea, BVD), bacteria (*Pasteurella multocida*, *Mannheimia (Pasteurella) haemolytica, Haemophilus somnus*) and mycoplasma spp. Many other risk factors contribute to progression of bovine respiratory disease. These factors include the environment (temperature, humidity, weather changes and dust particles) management (stress resulting from nutritional changes, weaning, shipping, vaccination, ventilation and stock density) and immunological background [2, 3].

Among these infectious agents, *Mannheimia haemolytica* is the major cause of acute pneumonia, also called Shipping Fever, in cattle [4, 5]. It is estimated to cause losses as high as one billion dollars a year to North American beef cattle industry [6]. *M. haemolytica* resides in the upper part of ruminant respiratory tract as an opportunist which causes acute pulmonary infection when the host immune system is suppressed by other factors. Infected cattle show typical clinical signs of BRD including fever, nasal

discharges, coughing, dyspnea, distress along with less appetite and weight loss. The infection with *M. haemolytica* results in rapid infiltration of neutrophils into alveoli together with fibrin and protein in the exudates. *M. haemolytica* components such as lipopolysaccharide (LPS) and leukotoxin in cooperation with neutrophil release of inflammatory factors cause damage to pulmonary parenchyma and alveolar epithelial cells [4, 5].

Additionally, lungs of cattle have a specific structure which limits their ability to resolve pulmonary diseases. Ruminants have less physiological gas exchange capacity but greater airflow rate and greater total lung volume for basal breathing than other animals. Small gas exchange capacity may lower the level of oxygen in the lungs, especially when the alveoli are obliterated by edema fluid or exudates. High ventilatory activity for compensation would be detrimental when cattle inhale contaminated air with infectious, noxious or airborne materials. Extensive interlobular septa in the lungs and interalveolar pores reduce the extent of alveolar expansion and collateral ventilation. These features predispose cattle to respiratory diseases [4, 7].

Bovine respiratory disease remains a major concern despite advances in genetic selection, vaccination, the use of antibiotics and anti-inflammatory drugs and other synergistic interventions. The thrust of current treatment modalities is to reduce inflammatory responses, control of airway reactivity and improvement of pulmonary functions without potential side effects [1].

1.2. Roles of neutrophils in acute lung inflammation

Neutrophils are the key contributors in acute lung inflammation [8]. The migration of activated neutrophils into the lungs as a result of increased alveolar capillary permeability along with production of a wide variety of inflammatory mediators contribute to elimination of invading pathogens. However, intensive and excessive neutrophil sequestration in response to stimuli can exacerbate the inflammatory condition [4].

The role of neutrophils in acute lung injury has been extensively studied in humans and animal models such as mice, hamsters and rabbits [8-12]. Neutrophil depletion prior to inoculation with *M. haemolytica* inhibits development of pneumonic lesions and protects calves against acute lung injury [13]. Elimination of neutrophils by antibodies against them significantly reduces endotoxemia- or hemorrhage-induced lung injury in mice [10]. During inflammation, neutrophils are activated by inflammatory mediators. These activated neutrophils generate a large amount of oxidants such as reactive oxygen (ROS) and nitrogen (RNS) species by the phagocyte NADPH oxidase and nitric oxide synthase (NOS); proteinases and peptides such as serine proteases and defensins. These products act as antimicrobial agents in the phagolysosome but are harmful to the host cells when released extracellularly [12, 14]. Apart from contribution to the redox signaling and microbial clearance, increased ROS impair oxidant-antioxidant balance leading to oxidative stress and consequent oxidative damage to pulmonary endothelial cells [15]. It is reported that neutrophil elastase triggers lung epithelial barrier dysfunction and increase alveolar edema by inducing apoptosis of these cells [12]. Due to deleterious consequences of excessive infiltration of neutrophils into alveolar spaces, modulation of

their migration is an attractive approach among treatment strategies of bovine respiratory disease.

1.3. Cell migration

Cell migration is an integrated process, which plays a key role in single-celled as well as in higher organisms. This process contributes to a large variety of biological phenomena starting from gastrulation and remaining during lifetime in normal physiological and pathological conditions as well. Migration governs fertilization, embryonic morphogenesis, contributes to cellular differentiation and specialization. Cell migration takes part in tissue repair, wound healing and regeneration, which involves migration of cells such as fibroblast, endothelial and epithelial cells. Remarkably, leukocyte migration in inflammation has been noted to modulate immune functions and contribute to the host defense by quickly moving from the circulation to target tissues in order to engulf pathogens, their debris and infected cells. However, dysregulated migration potentiates disease development in various chronic inflammatory diseases such as rheumatoid arthritis, multiple sclerosis and in tumor metastasis which give rise to many concerns. Therefore, many studies have been done to understand the mechanism of cell migration and to apply the understanding for the development of better therapeutics [16, 17].

Cell movement involves many factors including properties of migratory cells and their stimuli in the extracellular environment [16]. Cells are able to respond to various environmental changes that lead to different types of movements named chemotaxis, necrotaxis, galvanotaxis and haptotaxis [18]. Among these, chemotaxis is noticeably men-

tioned in leukocyte recruitment during inflammation. Chemotaxis is a cellular migration directed by gradient of chemoattractants in the extracellular environment [19]. It is distinguished from random migration by the presence of chemical stimulants.

1.3.1. Neutrophil directional migration (neutrophil chemotaxis)

Polymorphonuclear leukocytes (neutrophils; PMNs) play a crucial role as cellular components in the host innate immune system. They emigrate from peripheral blood to sites of infection in response to chemotactic factors derived from the host or invading pathogens [20]. The neutrophil migration is regulated by various proteins and signaling pathways and includes many steps such as rolling, tethering and adhering to the endothelium of postcapillary venules and finally crossing the endothelial barrier to reach extravascular tissues [21].

Neutrophils express various receptors for chemotactic stimuli which evoke neutrophil migration. Chemoattractants for neutrophils are derived from bacteria such as formylated bacterial peptides, fMLP (formyl-Methionyl-Leucyl-Phenylalanine), or from the host cells including endothelial, epithelial cells, macrophages, monocytes, lymphocytes, platelets, parenchymal cells and neutrophils. Chemoattractants not only attract neutrophils to move along their gradient but activate these cells to express adhesive proteins for migratory process, as well. Additionally, complement protein C5a and fMLP elicit potent activities such as cytoskeletal changes, degranulation, and oxidative burst [22].

1.3.2. Mechanism of neutrophil chemotaxis

Neutrophil chemotaxis is initiated by gradient sensing of chemoattractants in the environment followed by transmission of the signals across the plasma membrane through the binding of chemoattractants and their receptors expressed on neutrophils [19]. The receptor occupancy is dependent on chemotactic concentration [23]. Neutrophils sense chemoattractants via tyrosine kinase receptors and G protein-coupled (seven-transmembrane-helix) receptors [24-26]. It is interesting that neutrophils are able to amplify their responses to very little chemoattractant gradient at the early steps of gradient sensing. This is followed by polarization in which neutrophils turn into an asymmetric shape where the anterior and posterior parts of the cells differ [23, 27].

To migrate, cells must generate intracellular forces that lead them along the chemotactic gradient. The cytoskeletal asymmetry enables neutrophils to localize the filamentous actin (F-actin) at the leading edge of cells that leads to membrane protrusion and drives them to move forward. There are two structures which are rich in F-actin and responsible for protrusion: lamellipodia and filopodia [16, 17, 25]. While actin filaments form branching network in lamellipodia, they cluster into rope-like bundles in filopodia. Both types of polymerization are mediated by actin-binding proteins which play an important role to stabilize the actin polymer structure against the membrane. Of the many different signaling pathways proposed in regulation of the cytoskeleton, the Rho family GTPases have shown their critical role in many cell types. They consist of Rac, Cdc42 and Rho. GTPases are GTP binding proteins which are activated by GDP and GTP release dependent on guanine nucleotide-exchange factors (GEFs) [27]. Rac and Cdc42

play the central parts in actin polymerization and pseudopod formation at the leading edge of migrating neutrophils [28]. The actin polymerization and membrane protrusion generate tractional force in the direction of chemotactic gradient [16, 17].

At the back and sides of migrating cells, myosin II filaments play a crucial role in preventing the extension of pseudopods from the back of the cell and mediating contraction of the tail in order to translocate their cell bodies forward and detach the adhesion sites [24, 28]. The contractile force results from actin-myosin interaction and may involve other mechanisms related to contraction of filaments connecting to adhesive sites and polarized sensitivity [16, 17].

While actin filaments are believed to provide protrusive and contractile forces, other cytoskeletal components, microtubules, are responsible for movement of cellular organelles and proteins towards the correct orientation and maintenance of cell polarity [17]. However, microtubules show opposite effects on moving neutrophils to those in other cell types. Microtubules suppress neutrophil polarity in different ways which are related to Rho activation [29].

In support of membrane extension, adhesion structures are formed on neutrophils in order to facilitate their attachment to the substratum. They include selectins and integrins. Selectins are transmembrane glycoproteins including E-selectin, P-selectin and L-selectin, which are expressed by endothelial cells, platelets and neutrophils respectively. P-selectin is also expressed on endothelial cells. The family of selectins is responsible for initial tethering and rolling of neutrophils along the endothelium of the blood vessels [22, 30-32]. Membrane proteins including integrins are recruited towards the cell rim. Intracellular proteins such as talin bind to the cytoplasmic tails of integrins contrib-

uting to integrin activation. Once activated, integrins serve as a bridge connecting the extracellular matrix to the intracellular cytoskeleton. Adhesion is a critical step during cell migration through which cells gain traction to maintain motility [16, 17, 33].

1.3.3. Integrins

Integrins are heterodimeric transmembrane receptors expressed on a variety of cells in many animal species. They are comprised of two subunits α and β with an extracellular globular head, a long transmembrane region and a short cytosolic tail. There are 18 α (α1-α11, αE, αV, αIIb, αL, αM, αX, and αD) and 8 β (β1-β8) subunits that form 24 heterodimers in mammals [34, 35]. Integrins contribute to various fundamental cellular functions during development through linkage of cells and the extracellular matrix to facilitate cell adhesion, direct cells to their targets and transmit signals that mediate the growth, differentiation and survival of cells and tissues [33, 36].

1.3.3.1. The role of integrin signaling in neutrophil migration

Integrins play an important role in neutrophil migration, especially in the lung where neutrophil trafficking is substantially different from that in the systemic circulation. The number of neutrophils in alveolar capillaries is more than in systemic vessels while the role of selectins in neutrophil migration is less pronounced [37]. In contrast to the venular migration of neutrophils in organs such as liver, the capillaries are the site of neutrophil migration in the lung. Another difference is the contribution of the β2 integrin family [22]. They are 4 members including αLβ2 (CD11a/CD18; LFA-1: lymphocyte

function-associated antigen-1), αMβ2 (CD11b/CD18; Mac-1: macrophage-1 molecules), αXβ2 (CD11c/CD18) and αDβ2 (CD11d/CD18) [35, 38]. Neutrophil emigration into the lungs is mediated by two pathways with or without β2 integrin involvement dependent on various stimuli. Neutrophil migration is β2 integrin dependent when it is induced by *E. coli* and their endotoxin (LPS), *Pseudomonas aeruginosa*, immunoglobulin immune complex, interleukin-1 (IL1) and phorbol myristate acetate (PMA). In contrast, neutrophil migration into inflamed lungs can occur independently of β2 integrins in response to Gram-positive bacteria such as *Streptococcus pneumoniae*, group B *Streptococcus*, *Staphylococcus aureus*, hydrochloric acid, hyperoxia and complement protein C5a [22, 39, 40]. However, β2 integrin regulation of neutrophil migration can be altered not only by stimuli but inflammatory mediators produced by these stimuli and status of inflammation as well [41]. β2 integrins bind to their ligands, ICAM-1 (intercellular adhesion molecule), on activated endothelial cells following exposure to stimuli which induce β2 integrin-dependent neutrophil migration [39]. β2 integrins associated with their ligands mediate firm adhesion and transendothelial migration of neutrophils in response to chemoattractants. It is also believed that β2 integrins function in neutrophil attachment to the extracellular matrix [31].

Integrins, in addition to direct role in neutrophil migration, also initiate cell signaling. Integrin signaling occurs in two ways which are termed inside-out and outside-in. Integrins are normally expressed on the cell surface in inactive states with the bent conformation which prevents them from binding to their ligands. The low affinity can be changed through a process called inside-out signaling. This is initiated by changes of in-

tracellular signals following the cell activation induced by other factors such as chemokines and cytokines. In response to the signaling events, the cytoplasmic tails of integrins are separated from the ectodomains leading to unfolded conformation. The extended conformation of integrins makes them activated and induces high affinity for their ligands [33, 42, 43]. Conformational changes in the integrin length in turn initiate the outside-in signaling pathways. Unlike the inside-out signaling, the outside-in signaling requires collaboration with enzymes, proteins which regulate and/or assemble in adhesion structures. The outside-in signaling is initiated by integrin ligation with their extracellular ligands [44]. Following conformational changes, the integrins transmit signals through trans-membrane domains to cytoplasmic domains resulting in various subsequent responses.

Integrins influence cell migration in many species during development [36]. The activation of integrins with separated cytoplasmic tails initiates their interaction with the actin cytoskeleton through binding to proteins in the cytoplasm. Talin has a prominent place among a complex of proteins that interact with integrins. Talin connects the β cytoplasmic domain of integrins to cytoskeleton at the beginning of adhesive complex formation to obtain higher affinity for their extracellular ligands [45]. Once bound to the extracellular matrix, integrins are clustered on the cell surface, which promotes more association with the cytoskeleton. In turn, it is possible that more integrin clustering and reorganization of cytoskeleton and the matrix results in cell migration [46].

Integrin activation influences downstream signaling pathways that contribute to cell movement including focal adhesion kinase (FAK) and Ras/Rho GTPases. The FAK pathway is activated upon the integrin-ligand interaction. FAK, which is required for focal adhesions, is able to bind to integrin cytoplasmic domains, interact with cytoskeletal

proteins, talin and paxillin. The activation of FAK is further induced by interaction with Src kinase and through phosphorylation of two cytoskeletal proteins paxillin and tensin. Together with recruitment of other proteins, FAK activation is essential for cytoskeletal organization and mitogen-activated protein kinase (MAPK) activation [33, 45, 46]. Both Ras and Rho GTPases act upstream and downstream of integrin activation. These small GTPases, for example Rac and Cdc42, promote integrin clustering and this leads to their further activation and translocation on the cell membrane [47].

1.3.3.2. Role of αvβ3 integrin and its specific ligand, RGD peptides, in neutrophil migration

The αvβ3 integrin is one of the integrins involved in neutrophil migration. αvβ3 is expressed on many cell types including endothelial cells, platelets, smooth muscle cells, osteoclasts, tumor cells, monocytes and neutrophils [48, 49]. It has been found in several organs of dogs, pigs and cattle such as small intestine, kidneys, liver, skin, spleen, skeletal muscle and lungs [50]. In particular, integrin subunits αv and β3 have been shown in neutrophils and endothelium of rat acute inflamed lungs [51]. Ligation of αvβ3 integrin initiates various intracellular signaling pathways which are involved in physiological and pathological processes [49]. The heterodimer αvβ3 expressed on neutrophils is essential for their interactions with extracellular matrix (ECM) molecules and their interstitial mobililty [52, 53].

The αvβ3 integrin has been found at the leading edge of neutrophils migrating on vitronectin [54]. The integrin, however, is recruited and endocytosed at the rear end then

recycled to the front of the cells along with increase in cytosolic calcium concentration. These findings are consistent with the fact that integrin internalization is required for cell migration [55]. αvβ3 integrin constantly forms stationary focal adhesions to the substratum at the cell anterior within the zone of transient integrin clustering. The integrin in low density forms a tight bond to the cytoskeleton, which potentiates formation of lamellipodium. Rapid dispersal of integrins at the rear end of integrin clustering zone provides high density integrin focal adhesions and is regulated by depolymerization and collapse of the lamellipodial actin filaments. When the cell moves forward, the integrin in high density focal adhesions lose their firm cytoskeletal interactions at the cell posterior, start sliding and finally detach the substratum [56].

The αvβ3 ligands, RGD-containing peptides, are present in various extracellular matrix proteins such as vitronectin, fibronectin, fibrinogen and other proteins. The RGD (arginine-glycine-aspartic acid) sequence is recognized by integrin αvβ3 and other integrin heterodimers. However, the crystal structures of the extracellular domain of αvβ3 and ligand specificity contribute to their relatively high affinity binding. The ligand arginine binds to the αv subunit in a shallow groove while the aspartic acid side chain links the β3 subunit in the MIDAS region (metal ion-dependent adhesion site) through the metal ion. The two-site binding mode forms a bridge between the two subunits αv and β3 at the centre of the ligand-binding pocket. The glycine residue in the interface between the two subunits creates the hydrophobic interactions. The distance of arginine and aspartic acid side chains in the RGD motif that determines the high affinity to integrin αvβ3 is regulated by the ring closure, flanking amino acid groups and backbone confor-

mation. Furthermore, cyclization of RGD peptides also protects them against susceptibility to chemical degradation and improves their binding properties to the integrin [49, 57-60].

The aspartic acid of the cyclic RGD peptide plays an important role in formation of contact with both MIDAS and LIMBS (the ligand-associated metal binding site) ions during equilibration. Water in medium also contributes to the binding process with two molecules at the adjacent MIDAS and one at MIDAS ions. The coordination of these water molecules prevents access of other free water molecules to the tight coordination of the MIDAS ion and RGD loop aspartic acid. Additionally, electrostatic interactions between divalent cations and the positively charged arginine or negatively charged aspartic acid in the RGD sequence have a critical role in stabilization of the RGD-integrin $\alpha v\beta 3$ complex [57, 58]. These structural insights into ligand recognition by integrins are essential for improvement of RGD-targeting approaches.

Importantly, there is evident that integrins impact cell migration through regulation of the extracellular signal regulated protein kinase (ERK) signaling (*see details of signaling pathways in the following section)*. Ligation of integrins enhances autophosphorylation and activation of receptor tyrosine kinases (RTKs) which are required for the ERK activation. Furthermore, integrin engagement is essential for cytoplasmic cascades and translocation of the active ERK from the cytoplasm to the nucleus to modulate transcription which then induces and prolongs cell mobility consequent upon sustained MAPK activation and regulation of other activities such as cytoskeletal dynamics, organelle reorganization and integrin signaling [61-63].

1.3.4. MAPKs (mitogen-activated protein kinases) and neutrophil chemotaxis

Mitogen-activated protein kinases (MAPKs) are a family of kinases which phosphorylate substrates on the serine/threonine residues. They are involved in regulation of a wide variety of biological and pathological processes in vertebrates through contribution to cellular responses to various extracellular stimuli. These processes include cell differentiation, proliferation, transcription, metabolism, survival and motility [64]. MAPKs are differentiated by distinct motifs in their activation loops, consisting of extracellular-signal-regulated protein kinase (ERK/MAPK) with Thr-Glu-Tyr motif, p38 with Thr-Ala-Tyr motif, and Jun N-terminus kinase (JNK) with Thr-Pro-Tyr motif [65].

1.3.4.1. The ERK1/2 MAPK signaling

Two isoforms of the ERK are ERK1 (p44) and ERK2 (p42) that play critical roles in cell migration. They are activated through the engagement of integrins such as integrin $\alpha v\beta 3$ with their ligands and the binding of many growth factors and cytokines to tyrosine kinase receptors at the plasma membrane. These result in Ras activation followed by Raf-1 recruitment and subsequent phosphorylation of MEK1/2 leading to activation of ERK1/2 [61, 65]. The ERK1/2 can also be activated by Ras-independent pathways, which are involved in the Rho family GTPases, Rac and Cdc42, and their downstream effector PAK [62].

Activated ERK1/2 directly modulates cell migration through various pathways. Apart from the role in transcription, the ERK1/2 enables regulation of cytoskeletal dynamics and coordination with focal adhesion and microtubules in migrating cells [62, 66].

They phosphorylate myosin light chain kinase (MLCK), which enhances phosphorylation of myosin light chains (MLC) and consequently promotes actin polymerization and actin-myosin interaction. These events generate contractile force essential for cell migration [61, 65, 66]. Phosphorylated myosin II is important for the assembly of protein vinculin and zyxin at focal adhesions and in preventing membrane protrusion at the cell rim [67].

The ERK1/2 regulates cell motility through adhesion complex. They phosphorylate focal adhesion kinase (FAK), cytoskeletal protein, paxillin, and Ca^{2+}-activated proteolytic enzymes, calpains. These are essential keys for assembly and disassembly of adhesion complex during migration [65]. In addition, active ERK1 in association with $\alpha v\beta 3$ integrin is essential for effective function of this integrin during cell spreading on vitronectin [68]. Figure 1.1 summarizes linking of integrin ligation with intracellular signaling pathways that regulate neutrophil migration.

1.3.4.2. The p38 MAPK signaling

There are four isoforms of the p38 MAPK comprised of p38α, p38β, p38γ, and p38δ. The p38 is stimulated by many growth factors, cytokines and chemoattractants such as fMLP. Active p38 downstream activates MAPKAPK2/3 followed by phosphorylation of heat shock protein 27 (HSP-27) leading to reorganization of actin. The p38 also regulates adhesion dynamics through activation of paxillin and caldesmon [65, 69].

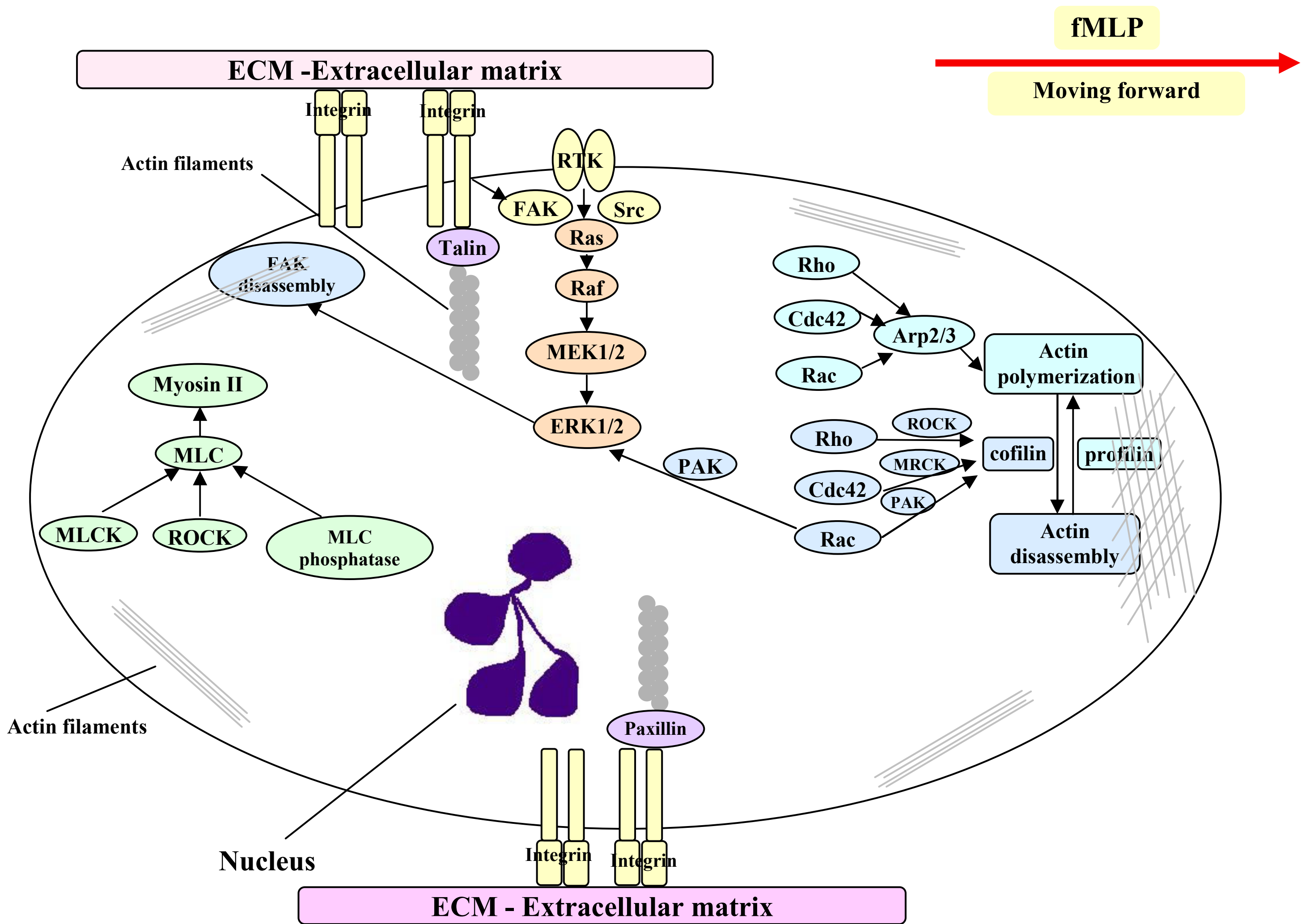

Figure 1.1. Integrin- and MAPK-mediated signaling pathways in cell migration.

Cell migration is initiated by polarization. Membrane protrusion at the front is mediated by the Rho family through assembly and disassembly of actin cytoskeleton. The Arp2/3 complex which is formed by regulation of activated Rac and Cdc42 facilitates branching of actin filaments causing membrane ruffles. Actin polymerization is regulated by two proteins with opposing effects: profilin (assembly) and cofilin (disassembly). Protrusion is enhanced by the formation of focal adhesion dynamics, a result of integrin engagement to the extracellular matrix (ECM). Integrins bridge the actin filament network and the extracellular matrix through cytoskeletal proteins, talin and paxillin. Ligation of integrins

enhances autophosphorylation and activation of receptor tyrosine kinases (RTKs) which are required for the ERK activation. Binding of growth factors to RTK activates Ras resulting in recruitment and activation of the Raf family at the cell membrane. Activated Raf phosphorylates the MEK1/2 kinase and downstream activates the ERK1/2. Active ERK1/2 in turn activates focal adhesion kinases (FAK) needed for detachment of cells from the ECM and moving forward. Another way of activation of ERK1/2 during adhesion is PAK, a downstream effector of Rac and Cdc42. At the back and sides of migrating cells, myosin II prevents the extension of pseudopods from the back and mediates contractile force for detachment. Myosin II activity is regulated by myosin light chain kinase (MLCK), which is phosphorylated by activated ERK, Rho kinase (ROCK) and MLC phosphatase [17, 62, 65].

1.4. Neutrophil apoptosis

After migrating to sites of infection and killing pathogens, neutrophils undergo spontaneous apoptosis. Apoptotic neutrophils are phagocytosed by macrophages in order to protect the host from noxious products of activated neutrophils such as proteolytic enzymes and reactive oxygen species [70].

Apoptosis is a process called programmed cell death, in which unwanted cells are genetically determined to be eliminated during development and homeostasis [71]. Apoptosis is characterized by morphological changes including cell shrinkage with condensed cytoplasm, rearrangement of cytoskeleton, chromatin condensation, nuclear membrane collapse, plasma membrane blebbing and formation of apoptotic bodies [72, 73].

1.4.1. Mechanism of apoptosis

Apoptosis is a complicated process modulated by two signaling pathways shown in figure 1.2: the extrinsic mediated by death receptors and the intrinsic dependent on mitochondria [73]. Extrinsic (death receptor-mediated) pathway is initiated by the binding of Fas death receptor to its ligand FasL. This leads to recruitment of adaptor protein FADD in association with procaspase-8 or procaspase-10 to form a death-induced signaling complex (DISC) at the plasma membrane. Caspase-8, activated by procaspase-8 autoactivation, directly activates caspase-3 and caspase-7, starting the execution phase of apoptosis [73, 74].

Intrinsic (mitochondrial-dependent) pathway is induced by various stimuli such

as toxins, radiation and free radicals. These stimuli cause changes in mitochondrial membrane resulting in release of proapoptotic proteins from the intermembrane space. The apoptotic protease activating factor-1 (Apaf-1) is activated by the release of cytochrome c from the mitochondria into the cytosol. The Apaf-1 binds to procaspase-9 in the presence of ATP to form the apoptosome for activation of caspase-9. Activated caspase-9 in turn downstream activates caspase-3, caspase-6 and caspase-7 [75, 76].

Both extrinsic and intrinsic signaling pathways end at the execution phase with the activation of execution caspases like caspase-3, caspase-6 and caspase-7. These caspases subsequently activate other proteases for degradation of nuclear and cytoskeletal proteins and substrate cleavage. Consequently, morphological features specific to apoptotic cells are revealed [73].

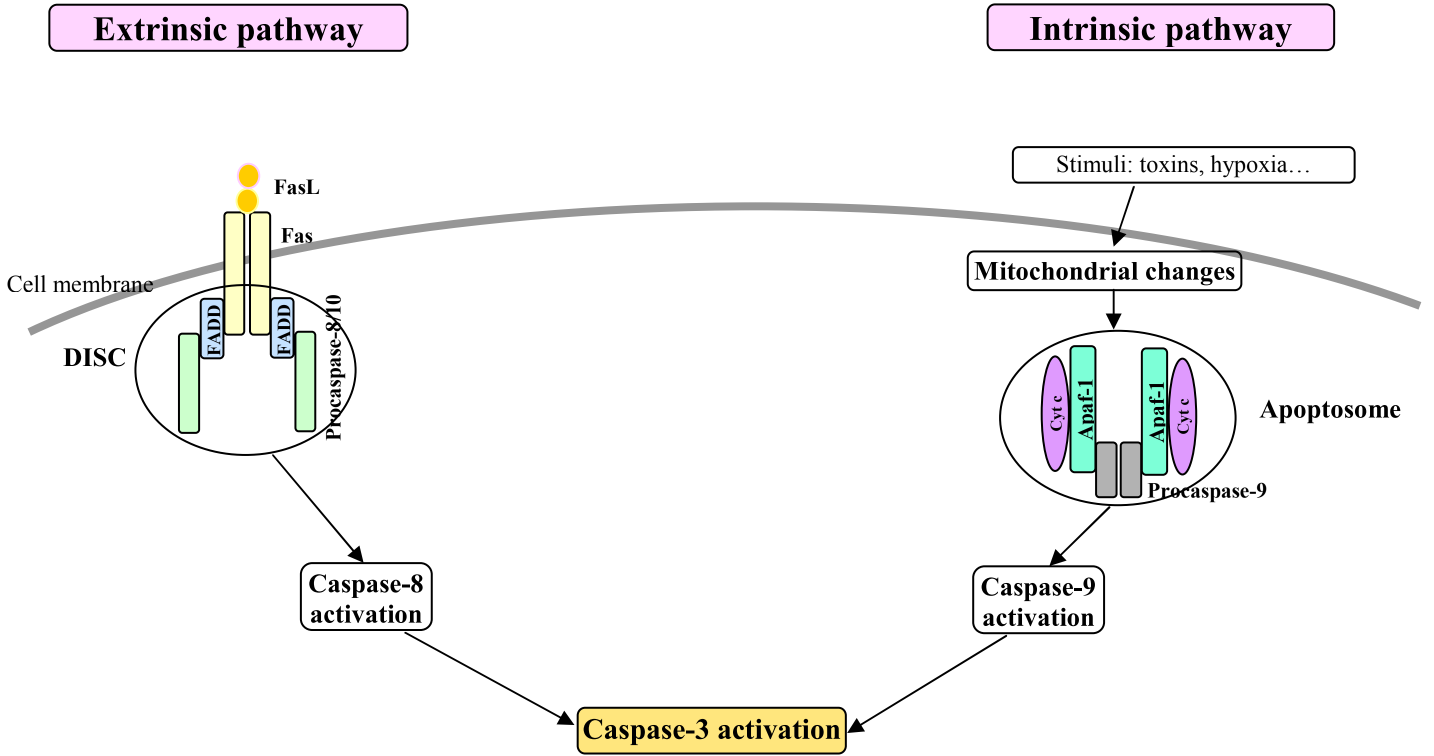

Figure 1.2. The two main apoptotic pathways: extrinsic and intrinsic [72, 73, 75].

1.5. Nanotechnology

Nanotechnology can be defined as a technology that develops and exploits the novel properties of materials at the nanometer scale [77]. According to the US National Nanotechnology Initiative (NNI), nanotechnology is also defined as "research and development aimed at understanding and working with – seeing, measuring and manipulating – matter at the atomic, molecular and supramolecular levels. This correlates to length scales of roughly 1 to 100 nanometers. At this scale, the physical, chemical and biological properties of materials differ fundamentally and often unexpectedly from those of the corresponding bulk materials" [78].

1.5.1. Nanomaterials

Nanomaterials are generic terms for nanosized materials, which have at least one dimension from 1 to 100 nm [79]. Nanomaterials exist in the natural environment for many years as byproducts of minerals, biological processes under influences of weathering, changes in climate and the earth crust [80, 81]. However, anthropogenic nanoparticles are increasingly introduced into the environment as a result of fuel combustion, urbanization, industrial emission and engineered nanoparticle production [82].

Nanomaterials exhibit large surface area compared to small size that gives them high reactivity or functionalization with other molecules [83]. Nanomaterials with exclusive properties are widely used in many products such as sunscreen, composite materials, paints, stains in textile industry and medical devices [79]. With enormous potential and great demand in various fields, nanomaterials are designed and functionalized with dif-

ferent materials that make them more specific and desirable for intentional approaches in electronics, energy and material production, optics, environmental purposes, medical diagnostics and therapeutics, and medicine [84].

1.5.2. Carbon nanotubes

Nanotubes together with nanofibers and nanowires are considered as nanomaterials with two dimensions in nanometric scale [84]. Carbon nanotubes (CNT) were first discovered in the 1950s for industrial application but they were not studied until 1991 [85]. CNT including diamond and graphite are members of the family of fullerenes, the third allotropes of carbon. CNT are composed of sheets of carbon atoms (called graphene) in a honeycomb-lattice structure that roll up into a cylinder with the two ends closed. CNT are categorized into two common types due to their structures: single-walled carbon nanotubes (SWNT) and multi-walled carbon nanotubes (MWNT). SWNT consist of one cylindrical sheet of graphene with diameters from 0.4 to 2 nm and lengths approximately in the range of 20 to 1000 nm. MWNT are comprised of several concentric grapheme layers with 1.4 – 100 nm diameters and lengths up to several μm [86-88].

CNT are functionalized by additional reactions or with surfactants, polymers and biopolymers that make them more soluble in solutions. Functionalization of CNT helps to improve their solubility and to reduce their toxicity for biomedical approaches. However, toxicity of CNT is dependent on types of CNT, methods and conjugated groups used for functionalization [87, 88].

1.5.3. Helical rosette nanotubes

Helical rosette nanotubes (HRN) are a new class of nanotubes which are self-assembled in water from low molecular weight synthetic modules [89, 90]. The formation of HRN is characterized by the self-assembly of the G^C motif, which mimics the complementary hydrogen bonding array of guanine and cytosine of DNA. G^C self-assembles into a six-membered supermacrocycle called rosette due to spatial arrangement of asymmetric hydrogen-bond network under physiological conditions. The rosettes are stabilized by 18 H-bonds and hydrophobic interactions that in turn self-organize into a helical stack with an approximate outer diameter of 3.5 nm, a 1.1 nm core and up to several μm in length (Figure 1.3) [89, 91-93].

Owing to biologically inspired features, rosette nanotubes have specific properties upon synthesis. Functional groups covalently attached to the G^C motif are exposed on the tube surface that give helical rosette nanotubes physical and chemical properties suitable for a variety of applications [93]. The stability of self-assembling tubes is dependent on several factors including density of functional groups, electrostatics (net charge), hydrophobic and stacking interactions [89]. The thermal stability of nanotubes is increased with more H-bonds per synthetic module and the organization of twin rosettes before building up a helical stack. Furthermore, the aggregation state of rosette nanotubes is pH dependent. At the low pH, these tubes are well dispersed while they form superhelices at the high pH [94]. This ability of helical rosette nanotubes to form different structures in biological solutions and systems remains unknown and is expected to be modified.

Together with development of nanobiotechnology, small cyclic peptides are in-

tensively studied and employed as biological molecules for nanotube functionalization. Specificity of binding sites and conformations of peptides give them selective affinity to carbon nanotubes [95]. The interaction of nanotubes and peptides provides specific tools for biological applications. Because of the critical role of neutrophil migration in inflammation and potential biomedical application of rosette nanotubes, we employed helical rosette nanotubes conjugated with RGD peptides to study bovine neutrophil chemotaxis *in vitro*.

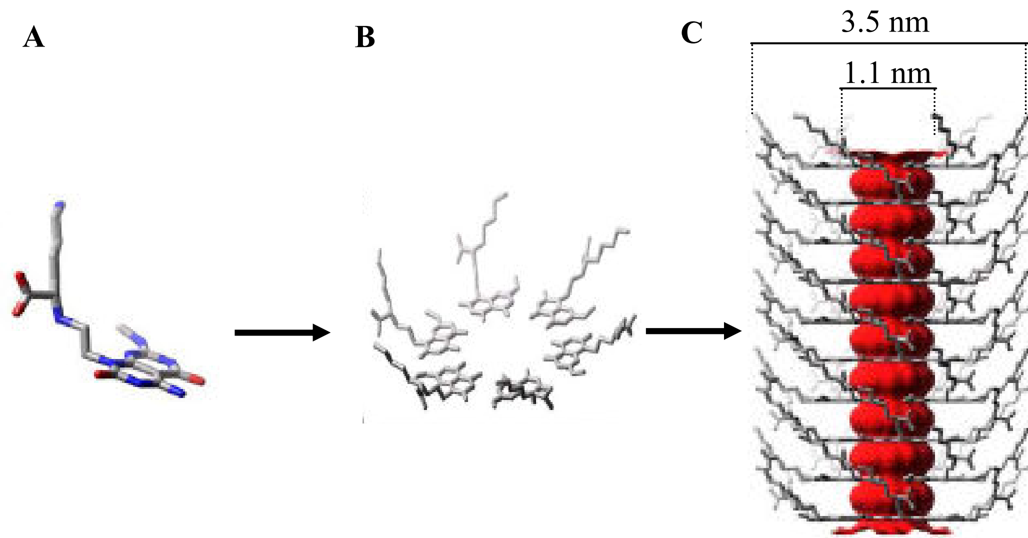

Figure 1.3. Helical rosette nanotubes. Modules (A) initially self-assemble into rosettes (B) which then organize into a helical stack with 1.1 nm core and a diameter of 3.5 nm.

2. HYPOTHESES

- RGD-rosette nanotubes (RGD-RNT) inhibit fMLP-induced neutrophil chemotaxis through binding to integrin $\alpha v\beta 3$ and inhibiting activation of the ERK1/2 and p38 MAPK.
- RGD-rosette nanotubes induce apoptosis in neutrophils.

3. OBJECTIVES

- To determine if RGD-rosette nanotubes inhibit neutrophil migration via binding to $\alpha v\beta 3$ and blockade of ERK1/2 and p38 MAPK.
- To study the apoptosis in normal and activated neutrophils after exposure to RGD-rosette nanotubes.

4. MATERIALS AND METHODS

4.1. Isolation of bovine blood neutrophils

Blood from healthy cattle was collected in vacutainer tubes containing heparin. Polymorphonuclear leukocytes (PMN, neutrophils) were isolated by density gradient centrifugation with lymphocyte separation media (LSM, MP Biomedicals) after using ammonium chloride for erythrocyte lysis [96]. After isolation, neutrophils were suspended in RPMI 1640 medium (Invitrogen) modified with 10% fetal bovine serum (FBS) and glutamine. The viability of isolated PMN was assessed immediately by trypan blue (Sigma-Aldrich®) exclusion using hemacytometer. Cell cytospin preparation was stained with Diff-Quik® and used for differential cell count. PMN viability was greater than 97% and their purity was more than 90%.

4.2. Neutrophil migration

4.2.1. Blocking the αvβ3 integrin by antibody

Isolated bovine neutrophils were suspended in modified RPMI-1640 medium, resting for 1 hour before any treatments. The anti-αvβ3 monoclonal antibody (mAb) specific to human αvβ3 integrin was purchased from R&D Systems. Neutrophils were incubated with the antibody at the concentration of 1 μg/ml for 1 hour at room temperature [97, 98]. The cells were then used for chemotaxis assay.

4.2.2. Blocking MAPK by MAPK inhibitors

Isolated bovine neutrophils were incubated with 20 µM of each MAPK inhibitors, either ERK1/2 inhibitor UO126 (Cell Signaling Technology, Inc.) or p38 MAPK inhibitor SB239063 (Calbiochem®), for 1 hour at 37^0C in humidified air with 5% CO_2. As UO126 and SB239063 were diluted in DMSO (dimethyl sulfoxide), DMSO was used as a negative control. The concentration of DMSO in all treatments was 0.2% (vol/vol) [99]. Subsequently, these cells were applied for chemotaxis assay.

4.2.3. Neutrophil chemotaxis assay

Chemotaxis of bovine neutrophils was assessed in 48-well Boyden chambers. The upper and lower parts of the chamber are separated by a 5 µm pore polycarbonate filter (Neuro Probe, Inc.) [100]. Neutrophil suspensions (50µl) with or without treatments were loaded in triplicate wells for each treatment with 5.10^4 cells per well. Cells in upper wells were allowed to migrate toward chemoattractant fMLP at the concentration of 114 nM in lower wells. The assay was conducted at 37^0C in humidified air with 5% CO_2 for 30 minutes. Non-migrated cells were wiped off the filter. Cells that had migrated and were stuck in filter pores were counted following drying and staining the filter with Diff-Quik® (Hemacolor stain set, EMD Chemicals). The stained filter was mounted on glass slides and cells within filter pores were then counted in 5 random fields under light microscopy at 400X magnification. Results are presented as the number of migrated neutrophils per microscopic field.

4.3. Detection of MAPK phosphorylation

4.3.1. Neutrophil stimulation with RGD-rosette nanotubes (RGD-RNT)

Isolated neutrophils were maintained in modified RMPI-1640 before incubation with RGD-rosette nanotubes (RGD-RNT; at the concentration of 0.1 μM RGDSK: 2 μM K1) in humidified air with 5% CO_2 at 37^0C at different time points at 0, 5, 10, 15, 30 and 60 minutes. fMLP (5μM) was also used as a positive control to induce phosphorylation of MAPK for 1 minute at 37^0C [101]. Stimulation was stopped by cell sedimentation and discarding of supernatants followed by freezing pellets in liquid nitrogen. Subsequently, cell pellets were stored at – 80^0C for later use.

4.3.2. Enzyme linked-immunosorbent assay (ELISA)

Cellular extracts were prepared by solubilizing pelleted cells at 5 x 10^6 cells/ml in lysis buffer comprised of 1 mM EDTA, 0.5% Triton X-100, 5 mM NaF, 6 M urea, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 100 μM PMSF, 3 μg/ml aprotinin, 2.5 mM sodium pyrophosphate, 1 mM activated sodium orthovanadate in PBS, pH 7.2 – 7.4. The protocol is recommended by the manufacturer (R&D Systems, Inc.). After vortex and ice incubation, supernatants were collected. Sample protein concentration was quantified using a protein microassay based on the Bradford dye-binding procedure (Bio-Rad). Cell lysates in duplicate for each time points were then used for sandwich ELISA (DuoSet® IC kit, R&D Systems, Inc.) to measure phosphorylated levels of ERK1/2 and p38 MAPK. Results are expressed by the amount of phosphorylated ERK1/2 or p38 MAPK (ng) per μg of total protein quantified.

4.4. Detection of neutrophil apoptosis

4.4.1. Cell treatments

Isolated neutrophils at 5 x 10^6 cells/ml were pre-incubated at 37^0C in humidified air with 5% CO_2 with or without LPS (lipopolysaccharide, 1 μg/ml). After 30 minutes, LPS was discarded and cells were resuspended in modified RPMI 1640. Cells were then treated with 0.1 μM of RGDSK peptide (an Arg-Gly-Asp-Ser-Lys containing peptide, Peptides International), 5% RGD-RNT (0.1 μM RGDSK: 2 μM K1) or 10% RGD-RNT (0.2 μM RGDSK: 5 μM K1) or modified RPMI 1640 only (control) for 18, 24 and 36 hours at 37^0C in humidified air with 5% CO_2. Cells were centrifuged at 400*g* followed by the removal of supernatant and snap freezing. The cell pellets were kept at - 80^0C for later use.

4.4.2. Caspase-3 quantification in apoptotic cells

Pelleted cells were applied for quantitative determination of caspase-3 using caspase-3 colorimetric assay kit (Assay Designs, Inc.). Cell lysates were used for caspase-3 colorimetric detection. Active caspase-3 in apoptotic cells converses a specific chromogenic substrate from colorless into colored product. The conversion was then measured kinetically at 405 nm. The activity of caspase-3 in samples was calculated as unit/ml.

4.4.3. Flow cytometric detection of apoptosis

For flow cytometry, the Annexin V-FITC apoptosis detection kit II from BD Biosciences was applied. Similar protocol was also described by Van Oostveldt et al. in 1999 [102]. Cells after 24 hours of treatments were collected, washed twice with cold PBS (phosphate buffer saline). They were then resuspended in 100 μl of 1X Annexin V binding buffer at the concentration of 1 x 10^6 cells/ml, following by adding 5 μl of Annexin V-FITC and 5 μl of PI (propidium iodide). The cell suspension was vortexed gently and incubated at room temperature in dark. Finally, 400 μl of 1X Annexin V binding buffer was added and cells were ready for flow cytometric analysis within one hour. Cells unstained with Annexin V-FITC and PI are live. Apoptotic cells in the early phase only stained with Annexin V-FITC while in the late phase they are stained with both Annexin V-FITC and PI. Results are expressed as the percentage of apoptosis.

4.5. Data analysis

Data was analyzed using SigmaStat® statistical software. All-pairwise comparisons were performed followed by analysis of variance (ANOVA) to compare differences between treatment groups. Results of at least 3 separate experiments are displayed as mean ± standard error of the mean (SEM). Differences are considered statistically significant when the probability $(P) < 0.05$.

5. RESULTS

5.1. Effect of RGD-RNT on neutrophil chemotaxis

Chemotaxis assays of bovine neutrophils were performed using 48-well Boyden chamber. Neutrophils in top of the chamber were allowed to migrate through filter pores towards chemoattractant fMLP in the bottom. Migrated cells stuck in the filter pores were stained and counted under light microscope. The modified RPMI-1640 was used in the bottom chamber to differentiate between directed (positive control) and random (negative control) migration. Neutrophils exposed to 5% RGD-RNT showed reduced random migration ($P<0.01$) as well as chemotaxis ($P<0.001$), compared to the non-treated group in control settings (Figure 5.2). Following exposure to 5% RGD-RNT for 5 minutes, neutrophils did not show significant increase in migration in response to chemoattractant fMLP.

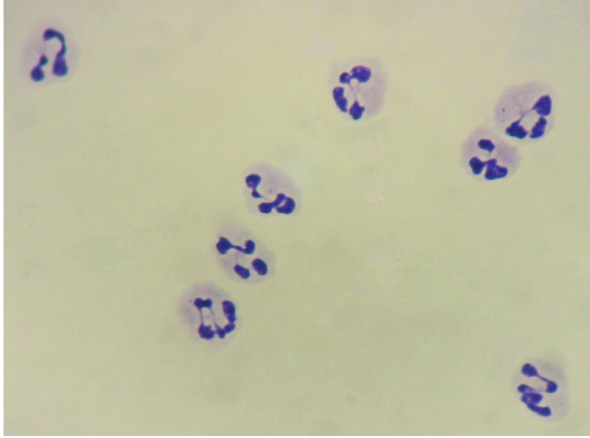

Figure 5.1. Bovine neutrophils isolated from heparinized blood.

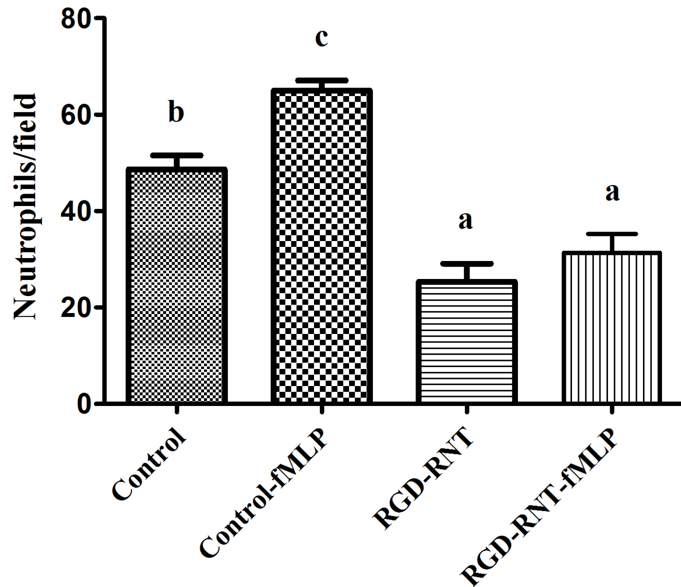

Figure 5.2. Effect of 5% RGD-RNT on bovine neutrophil chemotaxis.

Neutrophil migration was determined by counting the number of neutrophils stuck in filter pores after 30 minutes of chemotaxis assay at 37^0C in humidified air with 5% CO_2. Neutrophils significantly reduced migration after exposure to 5% RGD-RNT for 5 minutes, compared to non-treated group in control settings. Following exposure to 5% RGD-RNT for 5 minutes, neutrophils did not show significant increase in migration in response to chemoattractant fMLP. Modified RPMI-1640 and fMLP (114nM) in the lower chamber were used as negative and positive controls, respectively. Results are displayed as mean ± SEM of 3 separate experiments. Significant differences between treatment groups are expressed by different letters above bars (P<0.001).

5.2. Effect of RGD-RNT on MAPK phosphorylation

To understand effects of RGD-RNT on neutrophil migration, cells were exposed to 5% RGD-RNT at different time points and then their lysates were used to quantify the phosphorylated ERK1/2 and p38 MAPK by sandwich ELISA. fMLP was also used as a positive control to induce phosphorylation of MAPK. There was a significant difference between treatment groups with $P<0.001$ (the ERK1/2, Figure 5.3) and $P<0.01$ (p38 MAPK, Figure 5.4). The phosphorylation of both the ERK1/2 and p38 was inhibited at 5 minutes ($P<0.05$) of the RGD-RNT incubation followed by an increase at 10 minutes which was then sustained until 60 minutes.

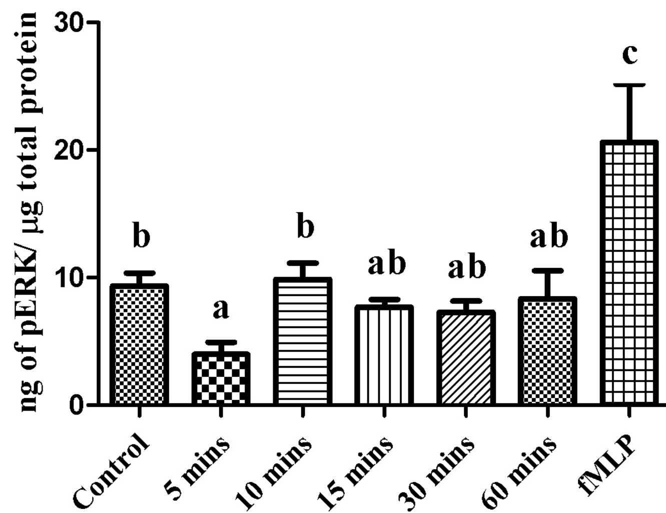

Figure 5.3. Phosphorylation of ERK1/2 in bovine neutrophils treated with 5% RGD-RNT at different time points. Phosphorylation of ERK1/2 was significantly inhibited at 5 minutes exposure to RGD-RNT. Non-treated and treated neutrophils with 5 μM of fMLP for 1 minute were used as negative and positive controls, respectively. Results of 3 independent experiments are represented as mean ± SEM. Significant differences between treatment groups are expressed by different letters above bars ($P<0.001$).

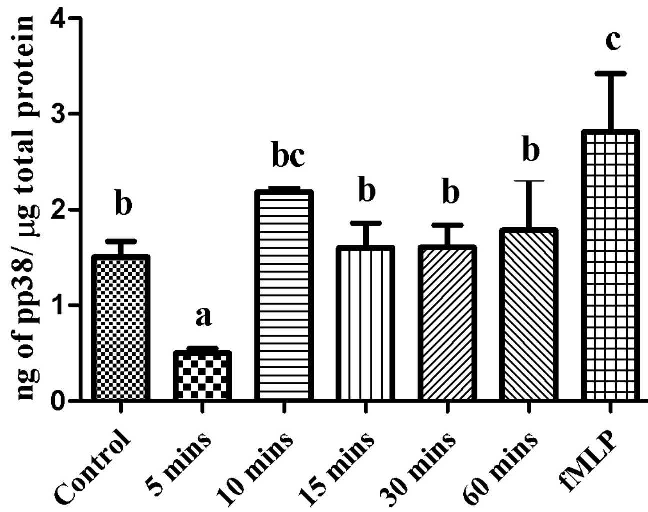

Figure 5.4. Phosphorylation of p38 MAPK in bovine neutrophils treated with 5% RGD-RNT in one hour time course. Phosphorylation of p38 MAPK was significantly inhibited at 5 minutes exposure to RGD-RNT. Non-treated and treated neutrophils with 5 μM of fMLP for 1 minute were used as negative and positive controls, respectively. Results of 3 independent experiments are represented as mean ± SEM. Significant differences between treatment groups are expressed by different letters above bars ($P<0.01$).

Neutrophils were also treated with the ERK1/2 inhibitor (UO126) and p38 inhibitor (SB239063) to assess the involvement of MAPK in their migration. The number of migrated neutrophils was significantly diminished ($P<0.001$) after exposure to these inhibitors. Intriguingly, the inhibitory effects of 5% RGD-RNT and MAPK inhibitors on neutrophil chemotaxis were not statistically different (Figure 5.5).

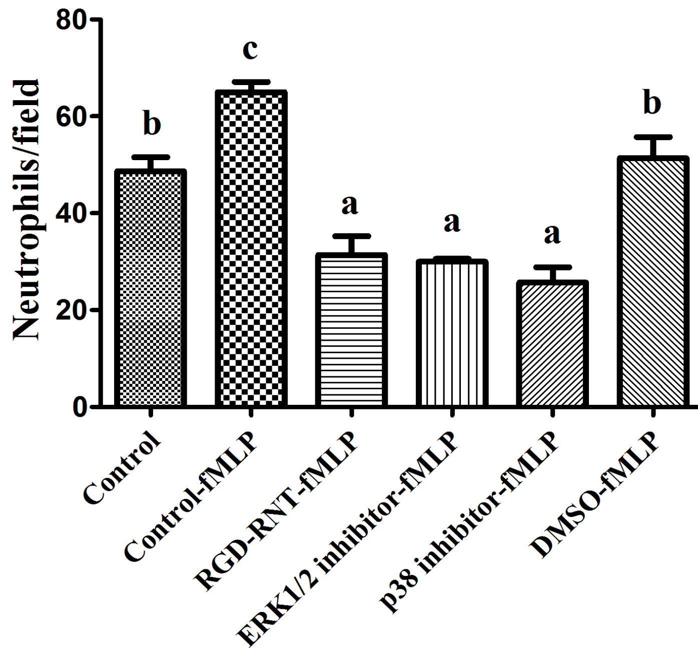

Figure 5.5. Inhibition of bovine neutrophil chemotaxis induced by 5% RGD-RNT or MAPK inhibitors. Neutrophil migration was determined by counting the number of neutrophils stuck in filter pores after 30 minutes of chemotaxis assay at 37^0C in humidified air with 5% CO_2. Neutrophil migration was significantly diminished after exposure to 5% RGD-RNT for 5 minutes or MAPK inhibitors for 1 hour. Modified RPMI-1640 and fMLP (114nM) in the lower chamber were used as negative and positive controls, respectively. DMSO (dimethyl sulfoxide), a solvent of MAPK inhibitors, was used as a negative control. Results of 3 independent experiments are displayed as mean ± SEM. Significant differences between treatment groups are expressed by different letters above bars ($P<0.001$).

5.3. Involvement of the αvβ3 integrin on bovine neutrophil chemotaxis

To determine the participation of the αvβ3 integrin in bovine neutrophil migration, chemotaxis assay was conducted with or without pre-incubation of neutrophils with monoclonal antibody (mAb) against the αvβ3 integrin. Migration of neutrophils treated with the αvβ3 integrin antibody was increased ($P<0.001$, Figure 5.6). Exposure of neutrophils to 5% RGD-RNT after incubation with the antibody had no effects on neutrophil migration in response to fMLP. There were no differences in the number of migrated neutrophils between groups with or without 5% RGD-RNT treatment after blocking by anti-αvβ3 mAb.

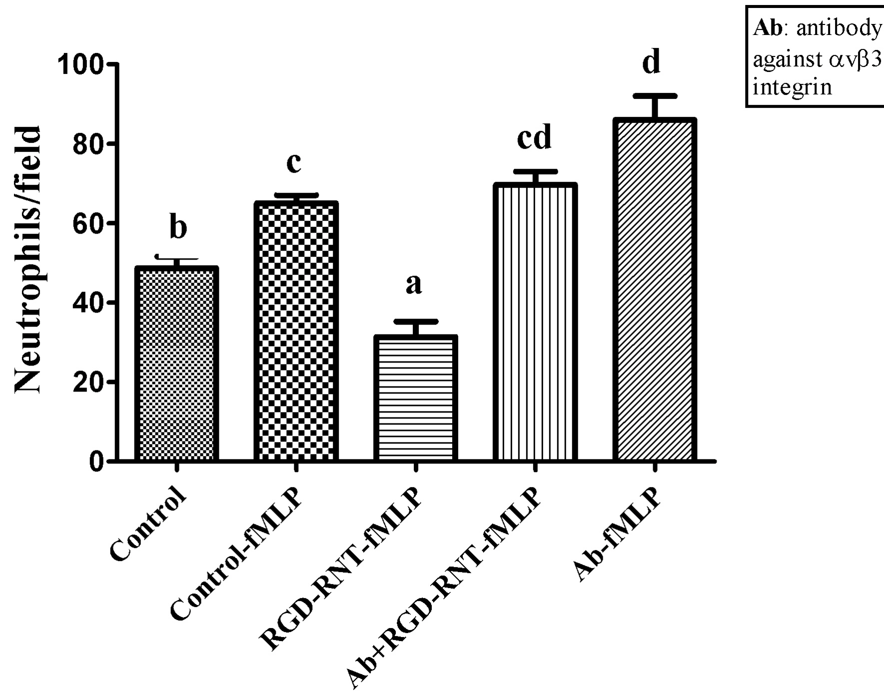

Figure 5.6. Effect of blockade of the αvβ3 integrin on bovine neutrophil chemotaxis. Neutrophil migration was determined by counting the number of neutrophils stuck in filter pores after 30 minutes of chemotaxis assay at 37^0C in humidified air with 5% CO_2. Neutrophil migration increased significantly after blocking by antibody against αvβ3 integrin. Exposure to 5% RGD-RNT after blocking the αvβ3 integrin by mAb had no effects on neutrophil migration to fMLP. Modified RPMI-1640 and fMLP (114nM) in the lower chamber were used as negative and positive controls, respectively. Results of 3 independent experiments are displayed as mean ± SEM. Significant differences between treatment groups are expressed by different letters above bars ($P<0.001$).

These results indicate that RGD-RNT at the dosage used inhibited the migration of bovine neutrophils. RGD-RNT also inhibited the phosphorylation of the ERK1/2 and p38 MAPK. The experiments show an intriguing role for the $\alpha v \beta 3$ integrin in neutrophil chemotaxis as the migration was increased following neutrophil treatment with the integrin antibody. The pre-treatment with the antibody also abrogated the anti-migratory effect of RGD-RNT, which suggests that both possibly bind to the same protein on the neutrophil surface.

5.4. Effects of RGD-RNT on bovine neutrophil apoptosis

Neutrophils were incubated without (controls) or with RGDSK peptide, 5% RGD-RNT or 10% RGD-RNT at different time points 0h, 18h, 24h and 36h. As shown in Figure 5.7, caspase-3 activity was increased over time in LPS-stimulated and non-stimulated neutrophils. However, the activity was not remarkably different over 3 time points namely 18h, 24h and 36h by the same inducible agent. The peptide and RGD-RNTs did not increase caspase-3 activity. But, 10% RGD-RNT significantly induced caspase-3 activity compared to the control at 18h ($P<0.05$). In addition, LPS caused significant suppression of caspase-3 activity in all treatments at all time points ($P<0.001$).

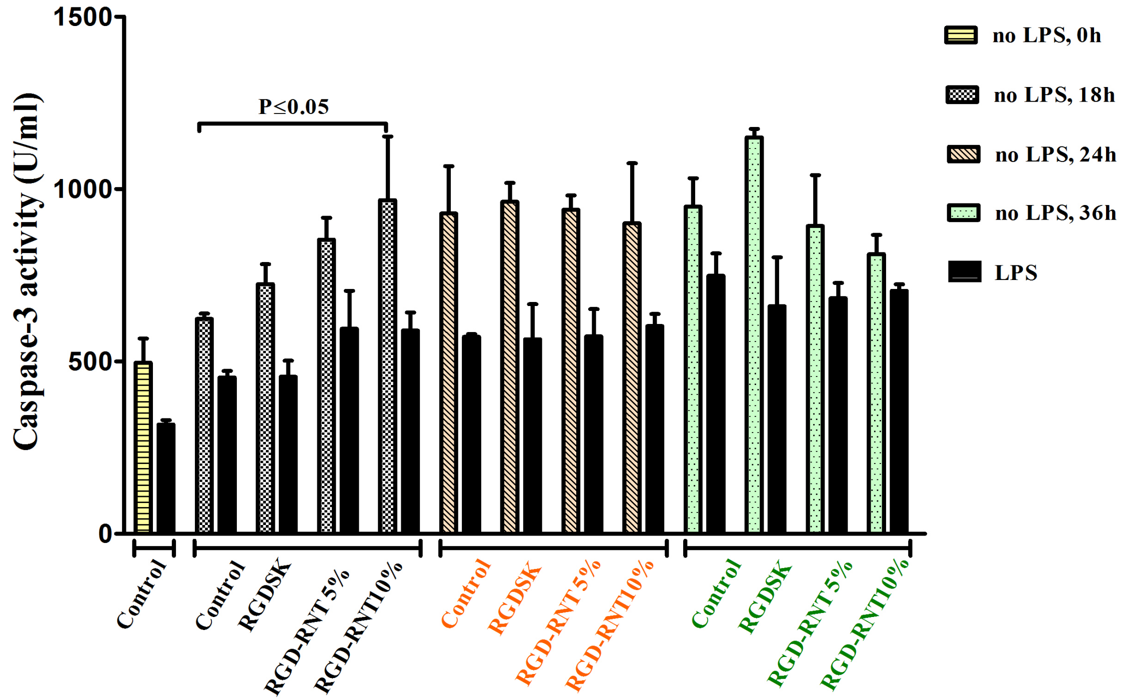

Figure 5.7. Effect of RGD-RNT on caspase-3 activity. Neutrophils with or without pre-exposure to LPS (1µg/ml) were treated with RGDSK, 5% RGD-RNT or 10% RGD-RNT for 0h, 18h, 24h and 36h. Cell lysates were used for measurement of caspase-3 activity. Results of 3 different experiments are displayed as mean ± SEM. P<0.001 when compared LPS and non-LPS treated groups.

The effects of RGD-RNT and LPS on bovine neutrophil apoptosis was also examined by flow cytometric analyses of labeling Annexin V-FITC which binds to phosphatidylserine expressed on cells undergoing apoptosis. There was no effect of treatment with RGDSK peptide, 5% or 10% RGD-RNT on neutrophil apoptosis at 24 hour. However, apoptosis was markedly suppressed in cells treated with LPS for 30 minutes ($P<0.001$, Figure 5.8).

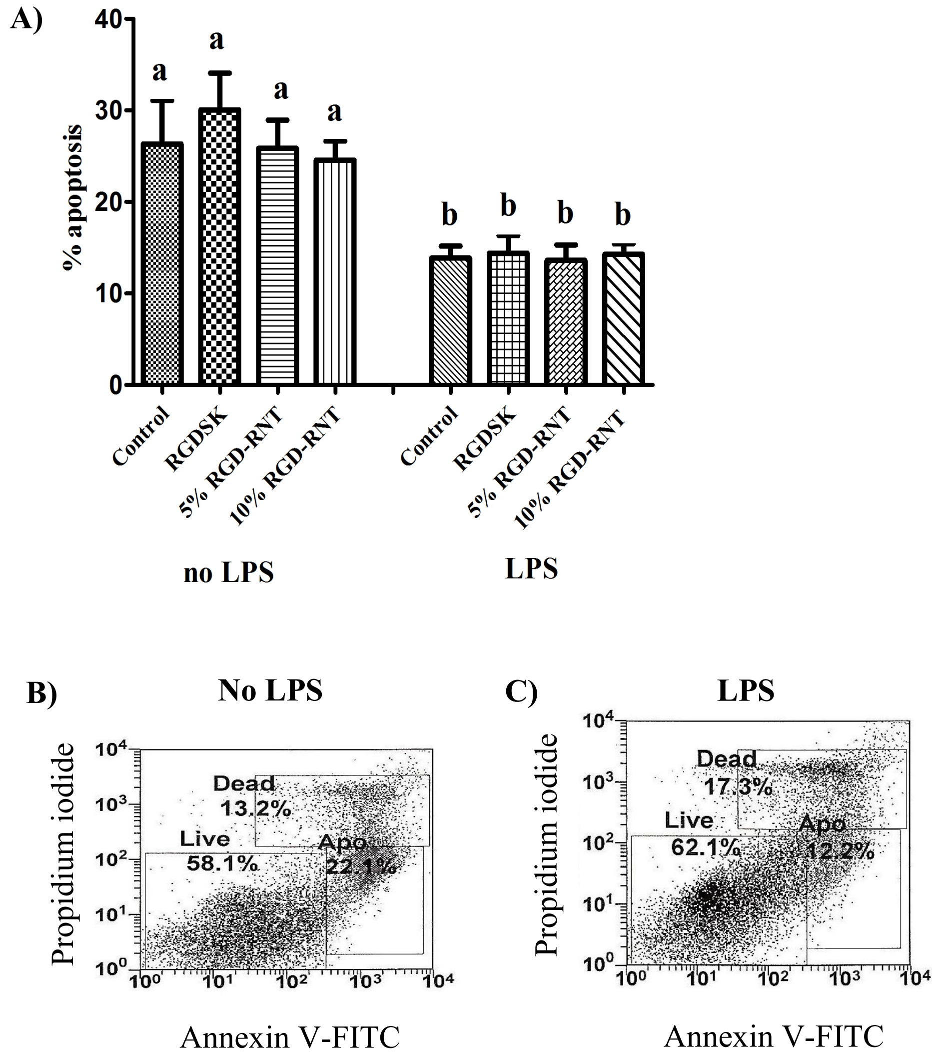

Figure 5.8. The effect of LPS on bovine neutrophil apoptosis at 24 hour in the presence of RGD-RNT. The percentage of neutrophil apoptosis is displayed as mean ± SEM of 5 separate experiments (A). The level of apoptosis was measured by Annexin V-FITC staining after 24h incubation without LPS-pretreatment (B) and with LPS-pretreatment (C). Significant differences between treatment groups are expressed by different letters above bars.

These observations indicate that RGDSK peptide and RGD-RNTs did not induce apoptosis or changes in caspase-3 activity of bovine neutrophils, except the induction of caspase-3 activity by 10% RGD-RNT at 18h.

6. DISCUSSION

6.1. RGD-rosette nanotubes inhibited neutrophil chemotaxis

Neutrophil migration into sites of infections is a critical component of the host response [103]. Activated neutrophils phagocytose and kill bacteria. However, there is growing evidence that dysregulated migration of activated neutrophils leads to tissue damage which results in morbidity and mortality [104]. This creates a need to exploit our understanding of mechanisms of neutrophil migration to develop approaches to fine tune their migration into inflamed organs. The motility of neutrophils is governed by integrin signaling as a consequence of interactions with their ligands in the environment [61]. In this respect, peptides containing RGD motif have been investigated to study cell adhesion and migration due to the recognition of this motif through integrins on the cell surface [105]. Cell migration is influenced by RGD and integrin clustering and their binding on the cell surface to form focal adhesions. Integrins have high affinity to RGD peptide even with their bent conformation which is considered an inactive conformation for other biological ligands [106]. Furthermore, integrins can efficiently bind to RGD ligands which are clustered on the surface at low density [107]. The binding of integrins to RGD motif causes conformational changes of integrins that in turn regulate integrin affinity to ligands and integrin redistribution on the cell membrane [108].

I conducted these experiments to study the effects of RGD-rosette nanotubes on neutrophil migration. The perceived advantage of RGD-nanotubes compared to cyclic RGD peptides is that the nanotubes may be highly effective at lower concentrations owing to their multivalent interactions with the integrins on the neutrophil surfaces. There is

some evidence that cyclic RGD peptides conjugated on nanoparticles are more active at the lower concentration than their monovalent states as peptides [109]. Helical rosette nanotubes have an added advantage of being organic, biologically inspired, water soluble and free of metal.

RGD-rosette nanotubes applied in this study inhibited chemotaxis of bovine neutrophils *in vitro*. The inhibition of human neutrophil migration has been observed when neutrophils were treated with flavoridin (FL), an RGD-disintegrin ligand of $\alpha v\beta 3$ and $\alpha 5\beta 1$ [110, 111]. Integrin ligands are able to trigger opposite effects dependent on their states in the extracellular environment. Non-immobilized ligands like RGD peptides act as inhibitors of cell adhesion while immobilized ligands promote cell adhesion to the extracellular matrix [105]. The other interpretation for inhibition of RGD-rosette nanotubes on neutrophil chemotaxis is desensitization. It can occur as a result of interactions between neutrophils and nanotubes. Migrating immune cells need to stop moving when they face target cells or invasive particles. Their responses to chemoattractants are desensitized by phosphorylation of chemokine receptors, G protein-coupled receptors (GPCRs) resulting from recruitment of G protein-coupled receptor kinases [112, 113]. Nevertheless, these are the first data on the anti-chemotactic effects of RGD-rosette nanotubes on the migration of bovine neutrophils.

6.2. RGD-rosette nanotubes inhibit the phosphorylation of ERK1/2 and p38 MAPK

I studied the activation status of MAPK as one of the possible mechanisms of

anti-chemotactic effects of RGD-rosette nanotubes. The binding of integrins and their RGD-ligand induces various intracellular signalings [46]. Of note, MAP kinase cascade regulates directional cell migration [65]. Activation of FAK (focal adhesion kinase) and c-Src is required for the MAPK activation by integrins. In turn, activity of the ERK is important for focal adhesion disassembly that leads to cell migration [62]. ERK1/2 has been found to modulate cell motility by direct phosphorylation of MLCK (myosin light chain kinase), which is required for myosin light chain (MLC) activity resulting in cell contraction and movement [114]. Because of the importance of ERK1/2 and p38 MAPK activation in neutrophil migration, I focused on these kinases.

The data from my experiments show significant suppression of the ERK1/2 and p38 MAPK activation after addition of RGD-rosette nanotubes to neutrophil suspensions for 5 minutes. It has been demonstrated that cell migration on fibrinogen is mediated by the $\alpha v\beta 3$ integrin through the ERK signaling pathway [115], and cyclic RGD peptides are considered as potent and selective antagonist of the $\alpha v\beta 3$ integrin [116]. Even though the ERK enable direct and rapid regulation of cytoskeletal dynamics in migrating cells, integrin ligation is required for induction and persistence of cell migration with regard to MAPK regulation [61-63, 66]. Considering the role of MAPK activation in cell migration, the reduction in MAPK phosphorylation at 5 minutes by RGD-rosette nanotubes suggests that the ERK1/2 and p38 MAPK may be involved in the inhibition of neutrophil chemotaxis by RGD-rosette nanotubes. There, however, is a need for further experiments to clarify the relationship of RGD-rosette nanotubes induced inhibition of MAPK phosphorylation and inhibtion of neutrophil chemotaxis.

6.3. Integrin $\alpha v\beta 3$ is involved in bovine neutrophil chemotaxis

In contrast to the inhibitory effect of RGD-rosette nanotubes, the monoclonal antibody (mAb) against $\alpha v\beta 3$ integrin increased neutrophil migration in response to fMLP. It was expected that blocking with anti-$\alpha v\beta 3$ mAb would inhibit the binding of $\alpha v\beta 3$ integrin on neutrophils and RGD peptides on rosettte nanotubes. However, mAb may regulate integrin-ligand interactions in different ways. The mAb, which recognizes epitopes present in the ligand-occupied sites, LIBS (ligand-induced binding sites), of integrins can induce conformational changes of integrins leading to ligand binding. Recognition of epitopes in the unoccupied sites, LABS (ligand-attenuated binding sites), conversely inhibits ligand binding [117]. On the other hand, the blocking mAb is thought to be endocytosed by an integrin-dependent pathway. The mAb together with $\alpha v\beta 3$ integrin are internalized in lysosomes. As a result, integrins on the cell surface are functionally blocked [118]. However, there is strong evidence that $\alpha v\beta 3$ integrin is internalized and recycled to the leading edge of neutrophils migrating on vitronectin [54].

It is possible that ligation of the $\alpha v\beta 3$ integrin by the antibody may have led to its cross-linking and consequent activation of cell signaling pathways resulting in increased neutrophil migration [44]. Furthermore, there are various integrins expressed on neutrophil surface that participate in cell movement. The activation of one integrin may cross-activate other integrins on the same cells [119]. Therefore, complete blocking of the $\alpha v\beta 3$ integrin by the mAb may also induce ligation of other integrins causing cell migration. Because pre-treatment of neutrophils with the antibody resulted in lack of effects of RGD-rosette nanotubes on neutrophil chemotaxis, it appears that both antibody and

nanotubes may be binding to the same protein, the αvβ3 integrin. These data, despite the need of more confirmation, implicate the involvement of integrin αvβ3 in neutrophil migration and RGD-rosette nanotubes may be acting through it.

6.4. Effect of RGD-rosette nanotubes on apoptosis of bovine neutrophils

The activated neutrophils compared to the normal ones live longer. The activation of neutrophils extends their lifespan by inhibiting constitutive apoptosis in normal neutrophils [120]. Caspase-3 is one of the critical enzymes involved in the terminal events leading to apoptosis [73]. Activation of caspase-3 is induced rapidly in apoptotic granulocytes without early mitochondrial changes [121]. Therefore, measurement of caspase-3 activity is necessary to detect early apoptosis in bovine neutrophils.

The cyclic RGD peptide is internalized by a fluid-phase endocytosis, which is not regulated by integrins [118]. Once getting into the cell, RGD is recognized via RGD-binding motif on procaspase-3 and some other caspases in the cytosol which then directly activates caspase-3 and promotes cell apoptosis [122]. It is consistent with our data that caspase-3 activity was increased in neutrophils treated with RGDSK peptide compared to the control over 36h time course. However, the activity of caspase-3 in RGDSK-treated group was not different from those exposed to RGD-rosette nanotubes.

Bacterial lipopolysaccharide (LPS) is believed to prolong neutrophil survival and hence hinder their constitutive apoptosis [123]. Prolongation of neutrophil survival is beneficial for the host resistance and regulation of inflammation; on the other hand, neutrophil activation causes more harm to tissues at sites of inflammation as a consequence

of production of inflammatory mediators such as reactive oxygen species [124]. Therefore, it is generally believed that we need to develop molecular approaches to regulate the lifespan of activated neutrophils.

My data show that treatment of bovine neutrophils with LPS for 30 minutes significantly suppressed caspase-3 activity in all treatments with or without RGDSK or RGD-rosette nanotubes at all time points. Exposure to RGDSK or RGD-rosette nanotubes did not induce the activity of caspase-3 except 10% RGD-rosette nanotubes which increased caspase-3 activity at 18h.

Percentage of neutrophil apoptosis at 24h was assessed by flow cytometric method using Annexin V and propidium iodide (PI) labeled with flourescein isothiocyanate (FITC) [102]. This procedure allows detection of apoptotic neutrophils at the early stage by staining with Annexin V, which binds to exposed phosphatidylserine (PS) in the outer plasma membrane of apoptotic cells. In addition, staining with PI, a vital dye, is able to distinguish cells in the early stage of apoptosis. At 24h, there was no significant effects of RGDSK peptide or 5% or 10% RGD-rosette nanotubes on neutrophil apoptosis compared to the control. However, significant differences were observed between LPS and non-LPS treated groups. This is consistent with many studies showing that LPS inhibits apoptosis of neutrophils [125-127]. Delayed apoptosis prolongs neutrophil viability and sustains their ability to eradicate pathogens by release of antibacterial factors, which in turn cause more tissue damage. On the other hand, LPS induces neutrophil necrosis that increases tissue destruction and persistence of inflammation [128, 129]. Indeed, LPS pretreatment statistically increased percentage of dead neutrophils while inhibited their apoptosis in all treatments at 24h ($P<0.001$).

This study showed that RGDSK peptide and RGD-rosette nanotubes at the concentrations used did not induce apoptosis or caspase-3 activity in bovine neutrophils except the induction of caspase-3 activity by 10% RGD-rosette nanotubes at 18h. In retrospect, I wonder whether using different concentrations of the RGD-rosette nanotubes or analyzing neutrophils for apoptosis beyond 36h would have shown effects of the RGD-rosette nanotubes as these tubes may need more time to manifest their effects in LPS-treated neutrophils.

7. SUMMARY

The first objective was to determine if RGD-rosette nanotubes inhibit bovine neutrophil migration. Incubation of neutrophils with 5% RGD-rosette nanotubes for 5 minutes reduced neutrophil chemotaxis to fMLP. This suggests the inhibitory effect of RGD-rosette nanotubes on bovine neutrophil chemotaxis.

To further understand molecular effects of RGD-rosette nanotubes on neutrophils, I assessed the phosphorylation of the ERK1/2 and p38 MAPK during an hour time course. The nanotubes reduced phosphorylation of both ERK1/2 and p38 at 5 minutes, which may be one of the mechanisms of action of RGD-rosette nanotubes on neutrophil chemotaxis. In addition, RGD-rosette nanotubes may bind to integrin $\alpha v\beta 3$ to affect neutrophil chemotaxis but more experiments are needed to clarify this aspect.

Finally, I examine neutrophil apotosis through measurement of caspase-3 activity and level of apoptosis. There were no effects of treatments with RGDSK peptide or RGD-rosette nanotubes on apoptosis and caspase-3 expression in bovine neutrophils, except an increase in caspase-3 activity by 10% RGD-rosette nanotubes at 18h.

8. CONCLUSION

In general, this study provides the first evidence that RGD-rosette nanotubes showed their inhibitory effects on bovine neutrophil chemotaxis and phosphorylation of the ERK1/2 and p38 MAPK. These bovine phosphorylated proteins, ERK1/2 and p38 MAPK, were quantified at the first time by capture ELISA. Additionally, blockade of anti-$\alpha v\beta 3$ resulted in more neutrophil migration which implicates the involvement of integrin $\alpha v\beta 3$ on neutrophil migration. Importantly, RGD-rosette nanotubes at the concentrations used in my studies did not induce apoptosis significantly and LPS dramatically suppressed the level of bovine neutrophil apoptosis.

9. FUTURE STUDIES

Based on the experiments described in this thesis, the following studies are proposed.

a. To study the specific role of the $\alpha v\beta 3$ integrin in bovine neutrophil migration.

b. To conduct experiments *in vivo* to assess effects of the RGD-rosette nanotubes on bovine neutrophil migration.

c. To understand the role of the $\alpha v\beta 3$ integrin in bovine neutrophil apoptosis.

d. To study the long-term effects of higher concentrations of RGD-rosette nanotubes on bovine neutrophil apoptosis.

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