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Review Article

Morphogen-induced Platelet Activation and Cell Signalling

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1. Introduction

Morphogens are signalling molecules that act directly on cells to produce specific cellular responses. This term was originally put forward by Turing (1952) who also described its chemical basis. Most of these secreted proteins signal between the cells. Wolpert (1969) first reported a role of morphogen in cell signalling. The roles of several morphogens (e.g., Transforming Growth Factor- β (TGF β), Hedgehog/Sonic Hedgehog, Wingless (Wnt) signalling, Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF), and Retinoic Acid (RA) in signal transduction have been studied by scientists in several cell lineages. Assoian *et al.* (1983) identified major storage sites of TGF β in human platelets. Vignani *et al.* (2003) showed expression of TGF β -regulated gene in a mouse mammary gland epithelial cell line.

Iwasaki *et al.* (2000) reported that EGF stimulates growth of vascular smooth muscles that may be involved in genesis of human vascular disease. RA acts as a ligand and diffusible morphogen, the distribution and levels of which are well controlled in embryonic tissues by its regulated synthesis and breakdown. The occurrence of RA signalling in embryonic development was subsequently reported by Rhinn and Dollé (2012). These cellular events play important roles in regulating the growth, proliferation and death of cells. They function in enormously diverse developmental processes, the dysfunction of which can lead to pathological consequences.

Platelets are unique cellular effectors of hemostasis and thrombosis. They are one of the important contributors to thrombotic disorders. These cells act rapidly in concert with coagulation proteins and other vascular cells. During biogenesis, platelets are generated as cytoplasmic buds from precursor bone marrow megakaryocytes. Therefore, they are morphologically devoid of nucleus and have no DNAs, and thus are incapable of transcribing nuclear material. In general, the transcriptional activity ramps up in individual megakaryocytes as they prepare to generate and release thousands of platelets (Patel *et al.*, 2005). Therefore, these terminally differentiated blood cells possess megakaryocyte-derived messenger RNAs (mRNAs) that have recently been demonstrated to be functional, leading to protein synthesis especially in activated platelets. Therefore, platelets are genetically predetermined cells and mRNA expression level within them is fixed. Thus, it would be interesting to study and elaborate the morphogen-induced cell signalling events within anucleate cells. The present article aims to highlight and review morphogen-induced cell signalling within platelets and it will be limited to the role of morphogens in platelet biology.

2. Morphogen Gradients and Platelets as Responding Cells

Morphogens are long-ranging signalling molecules that form a graded distribution across tissues to induce cellular responses and pattern development in concentration-dependent manner. Cells exposed to

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higher levels of morphogen molecules activate different transcriptional effectors, whereas those exposed to lower levels adopt different fates. Thus, the graded morphogen distribution subdivides tissues into distinct cell types, which are arranged as a function of their distance from the source (Briscoe *et al.*, 2010). Cellular response to morphogen is, therefore, mainly concentration-dependent.

Morphogens trigger signalling events through activation of receptors on their responding cells. Thus, expression of related receptors on or within platelets, which can respond to morphogen gradients and trigger platelet signalling would be highly impactful. Various studies have recently demonstrated presence of such specific receptors on platelet surface. For example, Lev *et al.* (2007) identified presence of functional TGF- β receptor on platelets. Steele *et al.* (2009) reported that Wnt signalling negatively regulates platelet function. In addition, role of platelet-derived growth factors (PDGF) in physiology and medicine is well described by Andrae *et al.* (2008). Moraes *et al.* (2007) put forward non-genomic signalling pathway of retinoid X receptor through binding and inhibiting G protein-coupled receptors (G_q) in human blood platelets. These studies have boosted an interest towards occurrence of morphogen-induced cellular events within human blood platelets.

3. Existence of TGF- β and PDGF as Morphogens for Responding Platelets

TGFs are biologically active peptides. They induce anchorage dependent, non-neoplastic cells to lose contact inhibition and undergo anchorage-independent growth. TGFs are classified into two types, type- α and type- β , based on their relationship with EGFs. Type- α TGFs are single chain, low molecular weight ($M_r < 10,000$) peptides that are defined by an ability to compete with ^{125}I -labelled EGFs for binding to EGF cell surface receptor. In contrast, Type- β TGFs do not bind to EGF receptor but they require EGF or TGF- α to express their biological activity. Type- β TGFs play essential roles in various cellular processes including early embryonic development, cell growth, differentiation,

motility and apoptosis in most cells.

Assoian *et al.* (1983) reported that platelets express 40-100 fold higher type- β TGFs than other non-neoplastic cells. Letterio *et al.* (1998) showed secretion of TGF- β by most immune cells including leukocytes. However, platelet-derived TGF- β , which is a polypeptide of 25,000 Da, possesses attributes that are different from other platelet proteins in many aspects. Conventional growth factors directly stimulate DNA synthesis to exert their effect whereas platelet-derived TGF- β acts via non-canonical pathway. TGF- β is weakly mitogenic whereas PDGF is a potent mitogen. Moreover, it is different from other platelet-associated chemokines such as connective tissue activating peptide-III (CTAP-III), platelet factor 4 and β -thromboglobulin. Due to these unusual biological properties, platelet-derived TGF- β is considered as a unique mitogen.

4. Existence of Wnt Signalling Pathway in Platelets

Multi-cellular organisms undergo activation of evolutionarily conserved signalling pathways, which include Wnts, Bone Morphogenic Proteins (BMPs) and Hedgehogs (Hh) during development. It occurs in a highly coordinated manner to ensure proper patterning of embryo. Upon release of these signalling molecules, cell populations induce intracellular signalling events in adjacent cells.

Wnt signalling plays a key role in maintenance of stem cell pluripotency during adult homeostasis. Wnts comprise a large family of secreted proteins acting as ligands. Logan and Nusse (2004) showed that Wnts affect diverse cellular processes leading to embryonic induction, generation of cell polarity and specification of cell fate. Wnt signalling is required in most embryonic developmental processes in both vertebrates and invertebrates. Wnt signalling has been classified into canonical and non-canonical pathways (Veeman *et al.*, 2003). In canonical Wnt signalling pathway, binding of Wnt to frizzled and LRP5/6 co-receptors leads to Wnt-dependent inhibition of glycogen synthase kinase-3 β (GSK-3 β) that results in stabilization of β -catenin and facilitates translocation of the stabilized β -catenin into nucleus

affecting transcription of target genes. Clevers and Battle (2006), Iwasaki *et al.* (2000) and Krishnan *et al.* (2006) independently observed that uncontrolled canonical Wnt signalling pathway is hallmark of cancer and other degenerative diseases. Therefore, regulatory mechanisms that control Wnt signalling are of crucial importance. In non-canonical Wnt signalling pathway, binding of Wnt to frizzled receptors does not involve GSK-3 β or β -catenin stabilization. Overexpression of ligands (Wnt5a or Wnt11) for non-canonical pathways causes an increase in intracellular calcium without affecting β -catenin level. It occurs due to activation of calcium/calmodulin-dependent kinase II (CAMKII) and protein kinase C (PKC). Some frizzled receptors can also activate heterotrimeric G proteins to activate phosphodiesterase and phospholipase C (PLC) that causes an increase in intracellular calcium level.

Platelet aggregation is a major process of hemostatic plug formation or thrombosis that occurs at sites of vascular damage in which platelets attach one another. Jackson (2007) demonstrated that several receptor mediated signalling mechanisms are involved in platelet aggregation. Platelets express several components of Wnt/ β -catenin pathway regulated by canonical and non-canonical Wnt signalling. Wnt3a and Wnt5a are ligands of canonical and non-canonical pathways respectively. Steele *et al.* (2009) found that Wnt3a inhibited platelet aggregation whereas Wnt5a was reported to be a stimulator of platelet aggregation (Kim *et al.*, 2011). Steele *et al.* (2009) demonstrated that Wnt3a ligand inhibits platelet adhesion, activation, dense granule secretion and aggregation. It also altered platelet shape change and inhibited activation of small GTPase RhoA. Thus, Wnt- β -catenin signalling pathway was revealed to be functional in platelets and can provide a target for future anti-platelet therapy. Moreover, the same group subsequently provided evidence that Wnt3a modulates platelet function by regulating activity of four key GTPase proteins, *viz.* Rap1, Cdc42, Rac1 and RhoA (Steele *et al.*, 2012). We have reported stabilization of β -catenin in activated platelets as important component of platelet signalling (Kumari and Dash, 2013). There we provided evidence that key canonical Wnt

signalling components are present in human platelets. Sustained aggregation of platelets for 15 minutes in presence of extracellular calcium was associated with nearly complete proteolysis of β -catenin. β -catenin cleavage was attributed to peptidase activities of calpain and proteasome, where integrin α IIb β 3 engagement and protein kinase C were other major players in stabilization of β -catenin. In another study, Kim *et al.* (2011) demonstrated that Wnt5a potentiates U46619 induced rat platelet aggregation via activation of PI3K/Akt signalling. Therefore, platelets respond to various canonical and non-canonical Wnt ligands in distinctive manner.

5. Epidermal Growth Factors (EGFs) and Platelet Biology

EGF is a naturally occurring molecule capable of stimulating cellular growth, proliferation and differentiation. It binds to epidermal growth factor receptor (EGFR) and further acts as a signalling molecule between cells. Human EGF is a 6045 Da protein that constitutes 53 amino acid residues and 3 intra-molecular disulfide bonds. They are present in human blood plasma, platelets, macrophages, urine, saliva and milk.

Oka and Orth (1983) have reported expression of human EGF in platelet-rich plasma, but not in platelet-poor plasma, using immunochromatography. They found human EGF predominantly in platelet fraction of whole blood. Platelet-associated human EGF is released into plasma during process of coagulation, thus implicating it in repair of tissue damage. EGF is expressed in platelets, especially at higher concentrations in alpha granules. Ben-erza *et al.* (1990) found that platelets neither have EGFR nor take up EGF from the medium. Megakaryocytes expressing EGF mRNA synthesize EGF and are the source of this factor in human platelets. Platelets provide a transport mechanism for several important tissue growth regulators to play a role in processes like atherosclerosis and tissue repair.

6. Retinoic Acid (RA) as Diffusible Morphogen and Platelets

RA is small lipophilic molecule derived from vitamin A. It stands as morphogen at distinct position among

other established morphogens. Marletaz *et al.* (2006) found that FGFs, Hh, Wnt, TGF- β bind to receptors present on plasma membrane of responding cells whereas RA enters nucleus and binds directly to target genes via nuclear receptors. Retinoid X Receptor (RXR) isoforms: alpha (α), beta (β) and gamma (γ) are three members of a family of nuclear receptors for RA. They are activated by 9-cis-retinoic acid (9cRA) and are present in a distinct tissue distribution pattern. For example, RXR type α (RXR $_{\alpha}$) is expressed in most of our body tissues but particularly higher in liver, kidney and skin, RXR type β (RXR $_{\beta}$) is found widespread, and expression of RXR type γ is restricted to heart and skeletal muscle. Moraes *et al.* (2007) reported that platelets express functional RXR $_{\alpha}$ and RXR $_{\beta}$ at both of its membrane and cytosolic fractions. They found that in spite of lacking nucleus, 9cRA (a RXR ligand) inhibits platelet aggregation to ADP whereas all-trans retinoic acid

(which is an isomer of 9cRA but not a ligand of RXR) does not exert any effect on platelet aggregation. Thus, RXR is considered pharmacologically active within platelets and not just a cytoplasmic remnant of megakaryocyte.

7. Conclusions

Morphogens have long been recognized as signalling molecules expressed ubiquitously in almost all cells in human body. The enigmatic roles of these small molecules to trigger signalling events within platelets have begun to be unravelled in the last decade. Morphogens act via transcriptional regulatory mechanism. Therefore, it is interesting to study their role and regulation in platelets, which are enucleate cells lacking DNA. Non-genomic effects of several transcriptional factors in platelets have begun to emerge. These novel pathways may provide valuable targets for future anti-platelet therapy.

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