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Baicalein inhibits agonist- and tumor cell-induced platelet aggregation while suppressing pulmonary tumor metastasis *via* cAMP-mediated VASP phosphorylation along with impaired MAPKs and PI3K-Akt activation

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ABSTRACT

Recently, the importance of platelet activation in cancer metastasis has become generally accepted. As a result, the development of new platelet inhibitors with minimal adverse effects is now a promising area of targeted cancer therapy. Baicalein is a functional ingredient derived from the root of *Scutellaria baicalensis Georgi*, a plant used intraditional medicine. The pharmacological effects of this compound including anti-oxidative and anti-inflammatory activities have already been demonstrated. However, its effects on platelet activation are unknown. We therefore investigated the effects of baicalein on ligand-induced platelet aggregation and pulmonary cancer metastasis. In the present study, baicalein inhibited agonist-induced platelet aggregation, granule secretion markers (P-selectin expression and ATP release), $[Ca^{2+}]_i$ mobilization, and integrin αIIbβ3 expression. Additionally, baicalein attenuated ERK2, p38, and Akt activation, and enhanced VASP phosphorylation. Indeed, baicalein was shown to directly inhibit PI3K kinase activity. Moreover, baicalein attenuated the platelet aggregation induced by C6 rat glioma tumor cells *in vitro* and suppressed CT26 colon cancer metastasis in mice. These features indicate that baicalein is a potential therapeutic drug for the prevention of cancer metastasis.

1. Introduction

Metastasis is a complex, multistage process and the main cause of about 90% of cancer-associated deaths [1]. Although many new therapies for treating malignant tumors have been developed over the past few years, the prognosis for most malignant cases remains unfavorable once metastasis has taken place. However, the mechanisms governing this clinically important process remain poorly understood and as a result, the treatment and prevention of cancer metastasis remains a major challenge.

Platelets are anucleate cells derived from megakaryocytes and are crucial for primary hemostasis as well as endothelium repair [2]. Platelets also play a role in pathophysiological conditions such as cancer metastasis. Recently, a great deal of evidence has been acquired in support of the notion that cancer metastasis is facilitated by the interaction between disseminating tumor cells and platelets during the early stage of cancer metastasis [3]. Blocking platelet/tumor cell interactions with anti-platelet agents strongly inhibits both spontaneous and experimental metastases [4]. Compounds directed against specific platelet

Abbreviations: TCIPA, tumor cell-induced platelet aggregation; ATP, adenosine triphosphate; ADP, adenosine diphosphate; IC50, 50% inhibitory concentration; cGMP, cyclic guanosine monophosphate; cAMP, cyclic adenosine monophosphate; MAPK, mitogen-activated protein kinase; VASP, vasodilator-stimulated phosphoprotein; PKA, protein kinase A; ERK2, extracellular signal-regulated protein kinase 2; αIIbβ3, integrin αIIbβ3; IBMX, 3-isobutyl-1-methylxanthine.

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receptors may thus give rise to new therapies for patients at a high risk of metastasis or for minimizing the risk of cancer cell dissemination during antitumor surgery. However, it is still unclear how platelet-mediated cancer metastasis can be suppressed without causing hemorrhagic or gastrointestinal side effects. For this reason, we have been engaged in identifying natural compounds from medicinal plants that exhibit anti-platelet activities.

Under normal conditions, collagen, the most potent thrombogenic factor, is sequestered under the endothelium of blood vessel walls. Disruption of the resting state of platelets begins with exposing the platelets to adhesive proteins in the subendothelial matrix at the site of vascular injury. Collagen that binds to the platelet receptor GP-VI initiates platelet activation, thereby promoting platelet adhesion to vascular endothelial cells [5]. A growing body of research supports the view that platelets are activated by collagen through a chain of cellular events more complex than that associated with other agonists [6]. Thereafter, the interaction of platelets with collagen promotes the cascades responsible for increasing the intracellular calcium ion (Ca^{2+}) concentration ($[Ca^{2+}]_i$), the mobilization of intracellular molecules, and enzyme phosphorylation.

Thrombin stimulates platelets by binding to the protease-activated receptors PAR1 and PAR4 [7]. This leads to further interactions between the platelets and the adhesive and soluble agonists that eventually initiate integrin αIIbβ3 inside-out signaling [8]. The role of thrombin in normal platelet function and the pathways that govern blood coagulation highlight the importance of this molecule as a link between the cellular (platelet) and biochemical (coagulation) causes of hemostasis [9]. Additionally, the importance of thrombin to metastasis *in vitro* and *in vivo* has been reported. In a previous study [10], thrombin increased the adhesion of platelets to a variety of tumor cells (B16, CT26, HCT8, and HM54). It was also reported that thrombin-treated platelets help regulate the metastatic potential of tumor cells (human rhabdomyosarcoma cells) [11]. In addition, thrombin-stimulated CT26 and B16F10 cancer cells showed significantly increased levels of pulmonary metastasis [12]. Thrombin-treated tumor cells not only interact with platelets to a greater degree than untreated cells, but they also show a greater capacity for adhering to endothelial cells [13,14]. Moreover, hirudin, a thrombin antagonist, inhibits 4T1 mouse tumor metastasis [15].

ADP is a secondary mediator of platelet aggregation that is released from platelet granules following activation and amplifies the initial hemostatic response. ADP binds to two G protein-coupled receptors (GPCR, Gq-coupled P2Y1 and Gi-coupled P2Y12) in platelets. The P2Y1 receptor activates phospholipase C-β (PLC-β) whereas the P2Y12 receptor activates phosphoinositide 3-kinase (PI3-K)/Akt and extracellular signal-regulated kinase (ERK), and inhibits AC activity [16]. The results of a previous study using selective purinergic receptor antagonists against the P2Y1 or P2Y12 receptor suggest that P2Y12 receptor is more important than P2Y1 receptor for tumor cell-induced platelet aggregation (TCIPA) [17]. Several tumor cell lines possess the ability to generate ADP and their potential for inducing TCIPA seems to be directly related to ADP production [18]. This indicates that the suppression of the platelet activation that is induced by tumor cell associated agonists may hold the potential for treating blood-borne cancer metastasis in the future [19].

Mitogen-activated protein kinases (MAPKs) control cell proliferation, differentiation, mitosis, survival, and apoptosis. Among these MAPKs, $p38^{MAPK}$, c-Jun NH_2-terminal kinase 1, and ERK2 are reported to exist in platelets and are activated by various stimuli [20]. Additional studies showed that collagen [21] and other agonists such as thrombin [22] and ADP [23] induce the phosphorylation and activation of ERK2. Furthermore, p38 and JNK are associated with collagen-mediated platelet activation [24,25].

The processes responsible for platelet activation are also controlled by cyclic nucleotide adenosine 3′,5′-cyclic monophosphate (cAMP) [26]. An increase in intracellular cAMP levels leads to the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) that suppresses fibrinogen binding to the integrin αIIbβ3. This suppression results in decreased agonist-mediated platelet activation, aggregation, and adhesion [27]. These data have provided a scientific basis for the potential use of cAMP-targeting agents as an anti-platelet therapy.

Platelet adhesion receptors play an important part in platelet/tumor cell crosstalk and hematogeneous metastasis [28]. The role of the integrin αIIbβ3 in TCIPA is also well known, and the significance of this integrin receptor has been demonstrated in many experimental tumor cell–platelet interaction models [29–32]. The importance of integrin αIIbβ3 to the interactions between tumor cells and platelets has been noted in several different tumor cell lines [33]. The *in vivo* importance of platelet αIIbβ3 in models of pulmonary metastasis was clarified by blocking integrin αIIbβ3 with the cognate monoclonal antibody 10E5 [33]. These findings were later confirmed by numerous studies [34–36]. Another adhesion receptor, P-selectin, is expressed on stimulated platelets or endothelial cells, and mediates the interaction among cells associated with metastasis, such as platelets, tumor cells, and endothelial cells [37].

Baicalein is a flavone found in *Scutellaria baicalensis Georgi*, a plant that has been widely used as a traditional herbal medicine [38]. Previous investigations showed that baicalein exerts chemopreventive effects against several types of cancer [39], and that its anti-inflammatory and cyto-protective activities are predicated on anti-oxidative and radical quenching properties [40–43]. Furthermore, baicalein has pro-apoptotic activities given its ability to induce Ca^{2+}-dependent mitochondrial dysfunction and to influence reactive oxygen species (ROS)-mediated pathways [44–47]. Although several previous works have demonstrated the anti-inflammatory properties of baicalein, the anti-platelet activities of this compound and the underlying molecular mechanisms are still not known.

In the current study, we evaluated the effects of baicalein on agonist-induced platelet aggregation *in vitro* and studied the associated molecular mechanisms. Our data showed that baicalein exerted a broad anti-platelet effect mediated through the inhibition of ERK2, p38, and Akt phosphorylation along with activation of PKA-dependent VASP phosphorylation. Furthermore, baicalein significantly suppressed TCIPA and platelet/tumor cell adhesion *in vitro* while preventing CT26 colon cancer cell metastasis to the lung *in vivo*. Our results suggest that baicalein may help prevent the aberrant platelet activation that is caused by tumor cell metastasis.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (7 weeks age, 240–250 g) and male BALB/c mice (7 weeks age) were purchased from Central Lab. Animal Inc. (Seoul, Republic of Korea), and were allowed to acclimate to a specific pathogen-free (SPF) laboratory animal facility with free access to water and feed (Purina, Seoul, Republic of Korea). All animal protocols used in the current study were reviewed and approved by the Institutional Animal Care and Use Committee at Dongnam Institute of Radiological & Medical Sciences (DIRAMS; Busan, Republic of Korea).

2.2. Materials

Thrombin, ADP, collagen, and ATP release kits for platelets were purchased from Chrono-Log (Havertown, PA). Baicalein, ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid