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Disintegrin-Like Peptides Derived from Naturally-Occurring Proteins: A Proposed Adjunct Treatment for Cancer Therapy: A Commentary

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Abstract

Disintegrins constitute a group of small proteins or peptides (45-85 amino acids) that function as natural antagonists of integrin receptor-dependent cell activities. The integrins themselves comprise a superfamily of hetero-dimeric (alpha and beta chains) transmembrane cell surface receptors whose functions include cell adhesion, growth, migration, and angiogenesis. In contrast, the disintegrins comprise groups of two types of molecules, namely, a) short proteins or peptides comprising insect and animal venoms; and b) intrinsic sub domain sequence fragments or short motifs present on large mammalian metalloprotease enzymes. Certain disintegrins bind specifically to tri-amino acid sequences (RGD, LGD etc) located on integrins beta-1 and beta-3 chains of the hetero complex receptors. Binding at such sites can inhibit or block cell migration, angiogenesis, metastasis, and platelet aggregation. Recently, small disintegrin-like peptides from naturally-occurring proteins have likewise been reported to inhibit growth and adhesion functions associated with integrin-dependent cell activities. The present report describes examples of such disintegrin-like peptides and provides support for their proposed use in adjunct cancer therapy.

Keywords: Disintegrin; Integrin; Peptide; Zinc; Metalloproteinase; Cancer; Clotting; Adhesion

Introduction

General

The containment of a class of internal growth modulatory peptide segments within a polypeptide chain of naturally-occurring proteins is a recurrent theme in cell regulation and signaling. Some of the more common growth inhibitors/suppressors consist of short amino acid sequence fragments derived from abundant plasma/serum, extracellular matrix, and angiogenesis-related proteins. Such fragment examples are: angiostatin derived from plasminogen; endostatin from collagen type XVIII; vasostatin from calreticulin, and tenecin from fibronectin [1]. While some segments are readily exposed on tertiary-folded protein surfaces, others constitute hidden, occult, or cryptic sites revealed following unfolding, denaturization, or proteolysis of the full-length protein [2]. The present study focuses on a cryptic site peptide fragment derived from a naturally-occurring fetal protein which is presented and discussed as potential disintegrin-like peptide (DLP) proposed for adjunct cancer therapies.

Types of Integrins and Disintegrins

The integrin superfamily of cell surface receptors consists of hetero-dimeric (alpha and beta chains) transmembrane glycoproteins that mediate cell-to-extracellular matrix (ECM) and cell-to-cell adhesion interactions [1-3]. In contrast, the disintegrins (DIs) exist in two forms, namely; a) families of free small proteins/peptides of 40-85 amino acids in length present in insect and snake venoms; and b) subdomain fragments or segments of a larger metalloproteinase enzyme; moreover, both such forms are capable of blocking integrin activities, interactions, and signaling [4-9]. Below, the animal venoms and protease subdomain DIs are discussed and compared to a disintegrin-like cryptic peptide derived from a naturally-occurring protein.

Objectives and Aims

The objectives in the present report are three-fold. First, biological functions and activities of the DIs are described. Second, the types and family members of the snake venom DI molecules are discussed together with their metallproteinase subdomain counterparts. Finally, an example of a disintegrin-like fetal peptide (DLP) is described which displays activities such as inhibition of platelet aggregation, cell to matrix adhesion, cell migration, spreading, and angiogenesis. It is presently proposed that such activities of DLPs might be harnessed to provide a novel source of adjunct therapeutic cancer agents.

The Disintegrins

The free single chain (DIs) disintegrins constitute families of small proteins and peptides ranging in length from 45 to 85 amino acids (AA) in insect and snake venoms. Many DIs serve and function as toxic

venoms that inhibit integrin-dependent activities such as: a) platelet aggregation; b) cell adhesion; c) cell signaling; and d) angiogenesis [8,9]. Other DIs comprise subdomain fragments inclusive on full-length metalloproteinase enzymes [10-12]. Such free and fragment DIs function by inhibiting the clumping or aggregation of platelets [13]. This action results in unimpeded bleeding and subsequent death to the host victim. The DIs bind and interact with the beta-1 and beta-3 chains of integrin receptors which normally serve as common pathways to aggregation *via* platelet-to-platelet interactions essential for thrombosis and/or hemostasis [14-16]. Certain DIs contain an RGD (Arg-GLy-Asp) or KGD (Lys-Gly-Asp) amino acid sequence motif that specifically binds to integrin IIb-IIIa receptors located on the surface membrane of platelets [17,18]. Other tri-amino acid single letter sequence codes include segments such as VGD, MLP, KTS, TRS, WGD, and RED [19]. The RGD-like binding event is capable of blocking the attachment of fibrinogen and von Willebrand Factor to a receptor-glycoprotein complex on platelets activated by aggregation agents such as ADP, thrombin, collagen, and platelet activating factor.

As discussed above, a wide array of true viper and pit viper venoms have been reported to consist of short peptide or protein disintegrins that block integrin-associated functions and activities [8,9]. Such or proteins peptides DIs are readily distinguished from the cobra-like venoms which are neurotoxins. Most disintegrins which contain the triamino acid or similar cell attachment recognition sequences are bordered by multiple cysteines resides [7,8]. Since disintegrins possess a variety of tripeptide sequences, they are further capable of inhibiting the adhesive functions of multiple integrins such as αvβ3 and αvβ5 (vitronectin receptors) and α5β1, the fibronectin receptor [13]. Thus, the disintegrins have been utilized to serve as models for designing novel and potent peptides for therapeutic use in the inhibition of platelet aggregation, blockage of the tumor-induced platelet aggregation stage of metastasis, and anti-angiogenesis during tumor development.

The second structural types of DIs consist of their presence as intrinsic subdomain sequence fragments on large metalloproteinase enzymes [20]. These proteinases comprise a family of enzymes referred to as ADAM (short for: A Disintegrin and Metalloproteinase) proteins. ADAM proteins are present either as single-pass fixed transmembrane proteins or secreted metallo-endopeptidase enzymes [21]. These peptidases exhibit multi-subdomain segments consisting of: a) a prodomain; b) a metalloprotease piece; c) a disintegrin subdomain; d) a cysteine-rich segment; e) an epidermal-growth factor-like motif; f) a transmembrane domain; and g) a C-terminal short cytoplasmic tail [21,22]. However, not all human ADAM proteins possess a functional protease domain suggesting a secondary role in protein-to-protein interactions for cell adhesion. ADAM proteins serving as active proteases can also cleave off and shed extracellular portions (subdomain) of their protein chain structure, such as an EGF-like fragment. The ADAM family members consist of at least 20 different types of metallo-protease enzyme members present on cells in many diverse organs and tissues.

Types of disintegrin proteins/peptides

The free single-chain disintegrins are derived largely from snake venoms such as Mojastin from Crotalusscutulatus, Salmonsin from Agkistrondon halys, and chistatin from Echis carinatus [23]. However, other DI-like peptides either free or intrinsic have been reported in mammals; these include: a guinea pig sperm (surface protein PH30), a mammalian epididymal apical protein (EAP-1), and intrinsic subdomain fragments from ADAM family proteinases such as ADAM-7, ADAM-10, and ADAM-17 [24]. DIs segments can be parsed into 5 different classes depending on the number of amino acid and cysteine residues in the peptide chain [25,26]. The first class includes the small DIs with 40-51 AAs and 4 disulfide bonds (DS). The second (medium) class of DIs displays 60-70 AAs with 6 DS bonds. The third (large) class contains at least 80-84 AAs and 7 DS bonds. The fourth dimeric class exhibits 67 or more AAs and 4 intra-chain DS bonds. Finally, the 5th type represent metalloproteinase subdomains containing 100 AAs and 8 DS bonds. Thus, DIs can include small to large free proteins/peptides, dimeric components, and subdomain segments from full length metalloproteinase enzymes.

Disintegrins and tumor-induced platelet aggregation

Tumor Cell-Induced Platelet Aggregation (TCIPA), a required component of metastasis, was first described by Gasic in the early 1970s [27]. Tumor cells in the blood vasculature are frequently observed complexed and clumped with platelets; this accumulation, together with the hypercoagulable state of the malignant disease, appears to be essential for successful metastasis. The ability of tumor cells to clump with platelets and induce platelet aggregation is widespread among cancers including breast carcinoma, colon adenocarcinoma, lung carcinoma, melanomas, and others [28,29]. A role for platelet participation in the metastatic process is thought to result from a) direct binding of platelets to tumor cells, and b) the release of soluble inducer agents from the tumor cells. These agents include the classical platelet aggregation activators such as ADP, cathepsin B, thrombin-like proteinases, collagen, and tissue factor-generated thrombin [30]. Thus, platelets act to facilitate the intermediate steps of transvascular metastasis and migration including tumor cell retention and arrest, subendothelial interaction, and extravasation from the micro-vasculature. Blockage at these steps can result in retarding or reducing migration of tumor cells during metastasis. Thus, the DIs represent a class of chemical agents whose therapeutic metastatic potential has yet to be fully realized.

Biological activities of Disintegrins (DIs) as anticancer agents

The cysteine-rich DIs bind to tri-amino acid sequence loops on Integrin membrane receptors located both on normal and cancer cell surfaces. Various receptor functions which can be inhibited by the DIs include activities of tumor cells associated with cell proliferation, adhesion, invasion, migration, metastasis, cell shape alterations, locomotion *via*-the cytoskeleton, and cell survival [8,9,11]. The inhibition of tumor cell growth by DIs is well-known being reported in multiple human cancer types such as; breast, pancreas, glioblastoma, lung, melanoma, colorectal, liver, stomach, kidney, and others [14,16,31-36]. The target cells to which DIs are attracted include epithelial cells, fibroblasts, white blood cells (i.e. neutrophils), ECM cells, tumor cells, and platelets.

DI inhibition of cell proliferation has been largely attributed to GO/G1 cell cycle phase arrest rather than apoptosis and/or necrosis. Some viper DIs, such as Tablysin-15, have been reported to suppress expression of cyclin-associated proteins such as CDK2, CDK6, Cyclin-D1, and Cyclin-E [17]. Certain snake venom DIs have been found to suppress the phosphorylation of focal adhesion kinases, the Akt protein, and beta GSK. Other DIs can block NF-κB nuclear translocation, while increasing the expression of the CDK inhibitor p21 (CIP) which halts G2-M cell cycle progression [34-37]. Interestingly, the increased expression of p21 alone can halt cancer cell growth, proliferation, and subsequent metastasis. Finally, snake venom DIs, such as "Viper anatolica" have been labeled with radionuclide intended for integrin-targeted radiotherapy to treat brain cancer [38].

Similar to the viper DI-like peptides, inhibitors of the zinc-endopeptidase subdomain fragments belong to families' of the metalloproteases such as ADAM transmembrane enzymes [39,40]. These DI-subdomains associated with metalloproteases are crucial participants in regulating the cell activities of cell-to-ECM adhesion molecules, cytokines, and growth factors. Thus, the importance of the disintegrin-metalloproteinase moieties lie in their modulating the ability of cells to control their extracellular environment, from remodeling of the extracellular matrix to the interaction of cells *via* adhesion and signaling in receptor cross-talk networks. The mechanisms of tumor cell growth inhibition of the ADAM-family proteinase subdomain fragments resemble those of the smaller protein or peptide venoms discussed above. The ADAM-DI subdomain binds to alpha-3, β1 integrin heterodimers, leading to cell cycle G1 phase growth arrest coupled with an enhanced expression of the cyclin-dependent kinase (CDK) inhibitor, p27 KIP1. This latter CDK inhibitor has been demonstrated to halt cell cycle progression in the G1 to S phase transition [41,42].

A Disintegrin-like peptide

Other than the PH30 and EAP-1 peptide/protein described above, a further example of and is integrin-like small peptide is the growth inhibitory peptide [GIP] segment derived from full length alpha-fetoprotein [AFP], a tumor-associated fetal protein [41,42]. The GIP segment is a 34-mer peptide sequence that lies buried in a molecular cleft of the tertiary-folded AFP polypeptide [43,44]. Following a conformational transformation, the intrinsic 34-amino acid presents as an exposed segment of human AFP which temporarily converts the growth-enhancing full-length AFP molecule into a growth inhibitory protein [45,46]. The transiently transformed growth-inhibiting AFP polypeptide present during pregnancy can temporarily halt growth until signal pathways can be repaired and restored in the fetus [47]. The transformed AFP molecule can subsequently refold into its tertiary native configuration which again conceals its growth inhibiting segment. The 34-amino acid GIP segment has been synthesized as a free peptide which has been purified and its biological activities characterized [45,48,49]. The free 34-mer GIP fragment has been reported to inhibit growth in breast and other human cancers in both *in vivo* and *in vitro* studies [50-52]. Interestingly, the GIP fragment demonstrates many of the activities displayed by DI peptides such as inhibition of platelet aggregation, cell/ECM adhesion and binding, and cell cycle arrest at the G1-S phase of the cell cycle (Table 1).

Activities of the disintegrin-like GIP

The AFP-derived peptide and platelet aggregation: Platelet Aggregation inhibition (PAI) has been studied using GIP in freshly prepared human platelet rich plasma preparations as previously reported [48,49]. The three platelet aggregation agonists used in these studies were Adenosine Diphosphate (ADP), Arachidonic Acid, (AA) and Collagen-II (Col-II) tested in platelet rich-plasma. The GIP fragment inhibited ADP induced platelet aggregation by 93%, blocked ADP collagen-induced platelet aggregation by 96%, and demonstrated 100% inhibition using AA. Thus, PAI occurred using all three agonists when the GIP segment was employed. Ristocetin, used a positive aggregation control, displayed 100% platelet aggregation in these studies. In summary, these data demonstrated that AFP-derived GIP was capable of PAI and that full recovery was achieved when Ristocetin was applied.

Cell adhesion assays using GIP: AFP-derived GIP was further subjected to cell adhesion studies using microtiter plates previously coated with ECM ligand proteins as attachment surfaces for two different breast cancer cell types: the human MCF-7 and the murine mammary 6WI-1 cell lines [48,49]. The adhesion of MCF-7 and 6WI-1 tumor cells, either in the presence or absence of GIP, was assayed on various ECM-coated microtiter plates. GIP was capable of inhibiting cell adhesion of the ECM ligand proteins in both tumor cell lines by 30-60%. Inhibition of mouse and human tumor cell adhesion was roughly equivalent in microtiter plates coated with either collagen IV, fibrinogen, fibronectin, thrombospondin, laminin, collagen-I, or vitronectin. As a result, human MCF-7 breast cancer cells in the presence of GIP, displayed 60% inhibition to vitronectin-induced adhesion, while mouse 6WI-1 cells demonstrated 40-50% peptide inhibition of tumor-to-laminin adhesion. Overall, the AFP peptide was found to competitively inhibit both MCF-7 and 6WI-1 cell-to-ligand attachment ranging from 40-60% in the various ligands employed [48,49].

Inhibition of cell migration, spreading, and metastases by GIP: Cell adhesion receptors and their ligands, (i.e., ECM proteins), provide the traction and stimulus for the migration and spreading of tumor cells both *in vivo* and *in vitro* [48,49]. Integrins mediate migration of adherent cells such as fibroblasts, epithelial cells, and tumor cells on ECM tissues and/or surfaces. Cell migration requires multivalent binding of integrins to matrix bound ligands such as collagen, laminin, and fibronectin [4,5]. Analysis of cell migration assays using GIP revealed that the peptide GIP was capable of inhibiting more than 60% of the MCF-7 cancer cells' spreading and migration on the surfaces of covers to be *in vitro*. The cells that inhibited migration displayed distorted morphology such as star-shaped configurations, cytoplasmic spiking, surface spiny spheres, membrane ruffling, and extensions of cytoplasmic processes; these events were coupled with low cell viability. It is important to note that cell migration and spreading constitute crucial steps in the cancer metastatic process; in this regard, GIP has been further reported to inhibit metastases in various animal models [44,49].

The AFP-derived peptides as anti-angiogenic agents: Angiogenesis, the formation of new capillaries from pre-existing blood vessels, is required for growth of solid tumors as well as in pregnancy, wound healing, tissue repair, placentation, and embryonic development [53]. The angiogenic process is composed of complex, multi-step stages encompassing four major events: 1) Cell migration, 2) proliferation of cells (endothelial), 3) cell survival, and 4) vessel tube assembly formation [54]. Thus, angiogenesis involves multiple cells, structures, and activities including ECM adherent cell activities, basement membrane alterations, cytoskeletal-induced cell shape changes, cell aggregation, receptor clustering, and ECM-to-integrin interaction [55]. Antagonism of the above constituents and associated events determine an agent to be an anti-angiogenic factor such as a disintegrin. Inhibition of these angiogenic events prevents the final assembly of endothelial cells into capillary tubes structures, i.e., tubulogenesis, which constitutes the endpoint of the angiogenic process. Moreover, without the first three stages of the angiogenic process (cell migration, proliferation, and cell survival), capillary vessel tube assembly cannot ensue.

The Chick Allantoic Membrane Assay of Egg Shell Membrane Vascularization

The Chick Allantoic Membrane (CAM) assay is a measure of blood vessel capillary structure formation on the inner membrane of the chicken eggshell [56]. Thus, the CAM assay serves as a rapid screening method of evaluating angiogenic agents in living systems. GIP was assayed for its anti-angiogenic properties in a tumor-angiogenesis

Table 1: A Comparative Listing of Activities and Traits Demonstrated by Free Disintegrins-Peptides and Protein Subdomain Components *versus* the Growth Inhibitory Peptide (GIP)*.

Activities, actions, traits, properties	Soluble and fixed Disintegrins	Disintegrin-like Peptides Example: Growth Inhibitory Peptides	
Platelet aggregation	Inhibits Platelet aggregation	Inhibits Platelet aggregation	48,49
Integrin Cell Adhesion	Inhibits/blocks cell adhesion	Inhibits/blocks cell adhesion	18,19
Angiogenesis	Inhibits angiogenesis	Inhibits angiogenesis	57-60
RGD binding recognition sequence	Contains, RGD, KGD tripeptide binding sequences	Recognizes RHE tripeptide binding sequence	22,45
Binds Integrin chains Beta-1 and Beta-3	Binds Beta-1 and Beta-3 chains	Binds Beta-1 and Beta-3 chains	4-6
Fixed and Soluble ECM Ligand Binding	Inhibits fixed and soluble Ligand binding	Inhibits fixed and soluble Ligand binding	5,6
Cell migration invasion and spreading	Blocks cell migration and spreading	Blocks cell migration and spreading	22,24
Cell/organism localization	Insect/snake venoms and ADAMS Family protein	Subdomain of oncofetal protein (alpha-fetoprotein)	7-9
Hemostatic status/cytoskeletal classification	Hemorrhagic, attaches to cytoskeletal proteins	Non-hemorrhagic, attaches to cytoskeletal proteins	11,12
Down Regulates expression of ADAM proteins	Changes of expression on ADAM 9, 10, 12, and 17	Down regulates expression of ADAM-22	58,54,60
Cell toxicity	Forms fusion toxins, induces hemorrhaging, binds integrin receptor	No known side ill effects; binds integrin receptor	33-35
Working concentration ranges	Nanogram to microgram concentrations	Nanogram to microgram concentrations	5,6
Cell targets	Integrin bearing metastatic cells, platelets ECM cells, neutrophil	Platelets, metastatic cells, cancer cells, ECM cells	9,10,60
Cell penetration and internalization	No known activity; no capability	Fusogenic cell penetrating peptide (microbial-like)	59,60
Anticancer activities and properties	Cytotoxic, cell detachment effect on Breast/other cancers	Cytostatic effect, inhibits cancer growth on (breast) multiple cancers	1-3,51,52
Platelet-to-cancer cell adhesion	Blocks platelet-to-cancer cell adhesion	Inhibits platelet-to-cancer cell adhesion	56,57
Effect on cell cycle progression	Induces G0 to G1 phase cell cycle arrest	Induces G1 to S phase cell cycle arrest	28,37,51
Unique peptide characteristic	Cysteine-rich and beta-hairpin loops	Cysteine-rich and beta-hairpin loop	38-40,51
Number of amino acids on peptide or protein subdomain	40 to 85 amino acid in length. (Viper snake Venoms)	Peptide of 34-36 amino acids in length	39,40,59
Tumor cell metastasis	Inhibits tumor cell metastasis	Inhibits tumor cell metastasis	50,51

Legend:*GIP derived from alpha-fetoprotein; ADAM=A Disintegrin and Metalloproteinase; RGD=Arg-Gly-Asp; KGD=Lys-Gly-Asp; RHE=Arg-His-Glu

model using the CAM assay in incubating chicken eggs [57]. In the CAM assay, mouse C5B1 melanomas were implanted on the egg shell inner membranes on incubation day 6 and observed 72 hr later for angiogenic vascular patterns which surround and attach to the tumor mass [49]. The results of these data demonstrated that GIP was capable of inhibiting of 95-100% tumor vascular growth induced by fibroblast growth factor (FGF) in the tumors which were transplanted onto the eggshell inner membrane.

Further Activities of Disintegrin-like Peptides

Recombinant and chimeric forms of DTs and ADAM subdomain fragments have been synthesized for use in studies of integrin inhibition of tumor growth, proliferation, adhesion, migration and angiogenesis of cancers such as liver, breast, lungs, and melanomas, [15,53,58,59]. In addition, the DI-like GIP has been reported to induce apoptosis in both radio-and chemo-sensitized cultured lymphocytes [49]. It has been further reported that ADAM-22 subdomain is an active participant in the development of breast cancer resistance during endocrine hormone therapy in women [59,60]. In lieu of this latter report, GIP administered to cultured MCF-7 human breast cancer cells for 7 days was shown to down-regulate the expression of ADAM-22 by 30-fold as determined by global RNA microarray analysis [51]. These data demonstrated that GIP treatment in MCF-7 cultured cells clearly down-regulated the expression of ADAM-22; in effect, this event might be capable of blocking the development of hormone-resistance in breast cancer cells.

Concluding Remarks

It appears plausible that interference with integrin signaling by DIs or DILs could provide a rational basis for the development of adjunct treatment modalities for cancer growth, progression, metastases, and angiogenesis. Anti-integrin antibodies, disintegrins- and DI-

like peptides have already shown promise in preclinical anti-cancer therapy studies. Integrin interruption of the adhesive interaction of tumor-to-tumor cells/platelets has been shown to arrest cancer growth progression and metastasis [14]. Disintegrin-like agents that block or interfere with the initial attachment of integrins to ECM components, might possibly blunt signal transduction events potentially inhibiting proliferation, cell migration/invasion, angiogenesis, and platelet aggregation. Such agents might serve to constitute a formidable armamentarium of non-toxic anti-cancer agents.

Since integrin dysfunction frequently results in cancer pathology, integrins represent an appealing set of targets for anti-tumor therapy. Because DIs specifically binds integrins, they could serve to interfere with and block integrin functions in cancer cell growth and proliferation as shown above. All such activities suggest that integrins could be viable candidates as molecular cancer targets and as such, make DIs and DLPs potential adjunct therapeutic agents to inhibit cancer growth and proliferation.

Conflict of Interest

The author declares that there are no known conflicts of interest in the preparation of this manuscript.

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