RGD-based Therapy: Principles of Selectivity

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Abstract: Design of selective anticancer agents targeting RGD-binding integrin receptors is known to be one of the perspective directions in molecular medicine especially in oncology. Significant progress in the development and application of such agents is already obvious. A great number of publications declare significant effects on both in vitro and in vivo experimental models. However the specific mechanism of action is generally not fully elucidated as well as the exact target responsible for the achievement of stated effects is not defined sufficiently well. To date 8 types of integrin receptors capable to recognize RGD-motif in natural ligands has been identified (namely αIIbβ3, αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, α5β1, α8β1). Even so the estimation of the affinity of one particular RGD-bearing anticancer agent is often based on the determination of the binding efficacy to only one or rarely two integrin receptors. Traditionally the range of targets is restricted by the integrins which are known to be highly expressed in a particular model system. While potential interactions of such an agent with other RGD-recognizing receptors usually remain beyond the research. Nonetheless such interactions may also affect the viability and behavior of cancer cells. In this paper we are going to review and discuss the principles of selectivity achievement in case of RGD-bearing natural ligands and the applicability of these principles in anticancer drug design.

Keywords: Integrin, RGD, anticancer therapy, drug, selectivity, auxiliary/synergy site.

1. INTRODUCTION

Selectivity is considered to be one of the major characteristics of anticancer drug design. Newly developed drugs should be able not only to recognize cancer cells but also to distinguish between cancer and normal cells. It has been attempted to force to look for an “ideal” target that is present only in the cancer but not in normal cells. Unfortunately, this strategy was unsuccessful so far. Very often researchers consider the molecules, which are overexpressed in cancer cells as compared with normal cells as a selective target.

It is well known that the integrin receptors are expressed in both normal and cancerous cells. While in certain cancers, the expression of the specific types of integrins is significantly increased. For instance, integrin αvβ3 is known to be highly expressed in several forms of the tumors such as osteosarcomas, glioblastoma, melanoma, neuroblastoma, lung carcinoma and breast cancer as well as on the activated endothelial cells (EC) of the tumor neovasculature [1]. These observations allow to hypothesize possibility considering integrins as selective potential targets for developing specific anticancer diagnosis and therapy.

Integrins are αβ heterodimeric receptors, which are responsible for cell-cell and cell-extracellular matrix adhesion. To date 18 types of α-chains and 8 types of β-chains have been identified [2]. Different combinations of α and β chains constitutes 24 types of integrin receptors. When interacting with cell surface or extracellular matrix integrin receptors may provide bidirectional transmembrane signaling and thus affect adhesion, cell proliferation, differentiation, growth, migration and viability [2].

Third of integrin receptors (namely αIIbβ3, αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, α5β1, α8β1) are known to be able to recognize RGD-motif in natural ligands such as fibronectin, vitronectin, fibrinogen, osteopontin, and tenascin. Several studies demonstrated that RGD-recognizing integrins are highly expressed in malignant cells [3]. For example, it was shown that the correlation between the expression level of integrin αvβ3 and melanoma progression [4]. Cells with high metastatic potential exhibit a 50- to 100-fold increase in integrin αvβ3 expression as compared to the cells with low metastatic potential [5, 6]. Based on these findings most likely potential possibility to use of RGD-binding integrins overexpressed in cancer cells can be specific markers of certain tumors. An ability of RGD-recognizing integrins to serve as oncomarkers on the one hand and at first sight as a simple structure of targeting tripeptide, while on the other hand, this seems to be attractive as featured in terms of development of highly selective for the diagnosis and anti-tumor therapeutics approaches.

During the last two decades a number of research groups investigated and attempted the possibility development of this type of selective agents. In earlier reports which have formed the basis of the field namely the conformation of tripeptide RGD was postulated as a main feature for effective and selective binding with the target receptor [7]. Later the crystal structures of integrin receptors in complexes with RGD-bearing ligands were determined and confirmed of the tripeptide in the binding pocket as the receptor was identified [8-10]. Afterwards, a set of molecules was developed which adopt the conformation close to the conformation of natural ligands in the binding pocket of integrin receptor [11-14]. Today, numerous RGD-bearing peptides of different origin and peptidomimetic are known to have an ability to selectively recognize and
bind to integrin receptors that overexpressed in cancer cells [14-17]. Nevertheless, so far developed and clinically approved RGD-based therapeutic agent most likely able to eliminate tumor cells in humans with high efficacy and strict selectivity still not yet available. What might be the reason of such misfortune? Maybe it is the fault of the course of events.

The development of a novel anticancer agent is often based on the improvement of the parent molecules, which were derived and characterized earlier [18]. That is why searching for target of such an agents is often restricted by integrin receptors, which were identified initially as a target of parent molecule, or by integrins, which are highly expressed in certain tumor cells. Therefore, the potential interactions of such an agent with other RGD-recognizing integrins, which are expressed at the similar level in tumor and non-tumor cells, usually remains beyond the research. Nevertheless, these integrins might still be capable for binding RGD-based agents and may determine following tumor cell reactions and viability.

So, the main question is: whether or not it is possible to design a strictly selective agent capable of direct action on only one certain type of RGD-recognizing integrin receptors? Thinking further on this matter prompted us to search for the mechanisms that ensure selectivity which are realized in the nature. Moreover, it has been established that natural ligands of RGD-recognizing integrins in humans possess different selectivity. Thus, fibrinogen preferably binds to integrin αIIβ3 [19], fibronectin - to α5β1 [20, 21], tenasin, can be bound preferably by integrins αvβ1 and αvβ6 [22]. The most preferable target for the foot and mouth disease virus (FMDV) capsid protein VP1 that is utilized by the virus for cell entry appeared to be integrin αvβ6 [23].

2. THREE-DIMENSIONAL STRUCTURE OF RGD-MOTIF

Development of tumor-directed agents bearing RGD tripeptide as addressing moiety often takes into account the conformation of RGD-motif. A number of studies aimed regarding resolving crystal structures of complexes of different integrin receptors with RGD-bearing ligands allowed to determine the conformation, which is adopted by RGD tripeptide in the binding pocket of integrin molecule.

It has been shown that even a single amino acid substitution in the tripeptide RGD violates its interaction with integrin αvβ3 [24]. Later on based on the X-ray analysis of the receptor-ligand complex it was found that such substitutions might lead to as to steric hindrance (replacement with larger amino acid residue, e.g., Asp → Glu) as to inability of formation of necessary chemical bonds for complex stabilization (due to the presence of side radical with another charge distribution, for example after the replacement of Arg → Lys) as well as to a failure to take proper conformation (due to the replacement of Gly → Ala). Arginine residue is located in the groove on top of the propeller α while the aspartic acid reacts both with βA and with Mn" ion located in MIDAS site. The glycine residue is located between α and β subunits and is involved in hydrophobic interactions with α [24].

After identification of the structural primitive of the minimal peptide capable of binding with RGD-recognizing integrins on the surface of tumor cells or activated EC of tumor neovasculature, a series of studies aimed to identify the structure of RGD-containing peptides possessing the highest specificity for the receptor and the lowest dissociation constant was performed. It was shown that the highest affinities were achieved in case of cyclic peptides [25] and in the case of polymeric peptides containing several RGD-monomers in its structure [26]. It was also shown that for effective interaction with the integrin receptor RGD-containing peptide should adopt certain bent conformation [27]. In the case of the cyclic peptides, such conformation is maintained by bending the structure in the ring, which explains better interaction with receptor than in case of linear peptides [28]. With regard to polymeric forms the presence of large number of the monomeric units increases the "local" concentration of the ligands in the vicinity of the receptor and the probability of reassociation events. This action most likely should give an advantage to polymeric forms in comparison with linear monomeric forms of RGD-containing peptides. The most interest to researchers are branched structures since theoretically subject to a certain distance between the integrin-binding structures they should be capable of binding a number of receptors simultaneously [29].

The importance of the structural aspect for effective RGD binding with the receptors was confirmed by a large set of data. First large group is short synthetic peptides in which cyclic peptides constitute a considerable part. One of the earliest was cyclic peptide cRGDfV, which was proposed as a non-toxic targeted agent for prostate cancer. It has been shown that the cytotoxic effect on αvβ3 expressing cancer cells with no effect on cells, which did not express αvβ3 [30]. Today the most advanced integrin antagonist in clinical development is Cilengitide™ (Merck, Germany). It is a N-methylated cRGDfV derivative c(RGDf(NMe)V) with high affinity to αvβ3, αvβ5 and αvβ1 [11-13]. It was shown to induce apoptosis in cancer cells in vitro by means of effective blocking its adhesion and migration mediated by αvβ3 and αvβ5 [12, 31]. In vivo Cilengitide™ reduced angiogenesis, tumor growth and metastasis [32]. More than twenty clinical trials involving Cilengitide™ have been completed and/or are ongoing, and the most notable were trials for the treatment of glioblastoma and prostate cancer [33, 34].

Another example is cRGDfK peptide, which is widely used as a targeting group to deliver different payloads. HPMA-copolymer docetaxel conjugates have been investigated in order to improve toxicity and solubility of current docetaxel formulations [35]. In vivo, αvβ3-targeted copolymer caused a significantly greater reduction in xenograft tumor growth in a breast and prostate cancer models if compared to docetaxel alone. In addition, cRGDfK peptide was also used to direct HPMA-copolymer geldanamycin conjugates to prostate cancer cells and tumor vasculature to avoid the dose-limiting toxicity of geldanamycin [36]. In vitro observation indicates that the inhibited HUVEC migration and tube formation whereas cRGDfK conjugate with geldanamycin or cRGDfK alone, and had no effect on HUVEC migration [37]. In vivo, αvβ3 targeting of geldanamycin increased intratumoral geldanamycin concentration over eight times than that provided by the equivalently tolerated dose of free drug, providing efficient inhibition of xenograft growth and angiogenesis together with doubling tolerable dose in nude mice [38].

Some of peptides form closed structures by means of disulfide bonds between cysteine residues flanking the RGD-motif. The RGD4c peptide is known to be one of the most prevalent αvβ3-binding peptide that used with the aim of visualization and impact on cancer. In particular, its potential in tumor angiogenesis imaging and targeted radiotherapy was demonstrated with the use of HPMA-copolymers chelating β-emitting isotopes [39, 40]. Numbers of fusion therapeutic molecules were designed on the basis of RGD4c. For example, a fusion protein containing a linear RGD peptides attached to the Fe fragment of mouse IgG has been used in gene therapy to target αvβ3 expressing tumors, and tumor angiogenesis through αvβ3 expressing EC. In vivo, injection of an adenovirus vector causing expression of the fusion protein significantly decreased the growth of DU-145 xenograft, and similar growth suppression was observed in tumors distant from the injected site [41]. While conjugation of the CD133GDFCFC peptide to IL-12 targets IL-12 directly to tumor neovasculature, this fusion protein stimulated interferon production in vitro and in vivo, suggesting biological activity - the result was significant enhancement of the antiangiogenic effect in corneal angiogenesis assay, augmented antitumor activity in a neuroblastoma model, and decreased toxicity of the IL-12 [42]. Linear CRGDC has been also shown to be effective in vivo if conjugated to the N-terminus of tachyplesin, a short antimicrobial cyclic peptide produced by the horseshoe crab. RGD-
tachyplesin triggers apoptosis in cancer cells and inhibit TSU xenograft growth in the CAM model without toxicity to the embryo [43]. In some reports the effective interaction of CRGDC-containing peptides with murine and human melanoma cells and with EC of tumor neovasculature has been also demonstrated [44, 45]. Finally, recently identified peptide iRGD (CRGDKGPDC) is able to specifically recognize and penetrate cancerous tumors but not normal tissues [46]. That is why it has been used to promote tumor penetration of imaging agents and drugs. iRGD contains a cyclic RGD motif which first binds to tumor cells via αv integrins and then undergoes proteolytic cleavage to reveal a linear CendR fragment with affinity for neuropilin-1, which allows cell penetration. iRGD has high affinity to αvβ3 and αvβ5 but not α5β1 and is localized to the regions overexpressing both αv and neuropilin-1 in 22Rv1 orthotropic prostate cancer xenograft. iRGD-coating of abraxane increased abraxane accumulation in 22Rv1 tumors eightfold compared to the untreated drug, whereas CRGDC-targeting only afforded a two-fold increase, and effectively inhibited tumor growth. Additionally, conjugation to iron oxide nanoworms allowed MRI imaging of the whole tumor region, a significant improvement compared to the CRGDC peptide which only targeted tumor vasculature and/or untreated nanoworms which gave no signal in the tumor [47]. Use of iRGD for drug delivery provides a substantial advantage over other integrin-targeting peptides due to its greater tissue penetration promoting capabilities. These were examples of synthetic peptides and proteins, but binding to integrins as we already mentioned depends on structure but not on the nature of the molecule. Therefore peptidomimetic, which do not have an RGD sequence in their structure but adopt the conformation similar to the conformation of RGD-bearing ligands of integrin receptors, are also shown to be capable of efficient binding to different integrins [14-17]. While developing integrins targeting agents, all attention of the researchers is generally focused on the affinity of binding with target integrin receptor. Indeed, detailed investigation of the binding abilities of the aforementioned molecules demonstrated highly effective binding with appropriate integrin receptors. However, the question of selectivity of these molecules is still open. The RGD structure is highly conserved and located in integrin-binding loop of almost all ligands of RGD-recognizing integrins. Similarly, the amino acids constituting the RGD-binding pocket of integrin receptors are conservative among different integrins. Indeed, the α5β3 and αvβ3 integrins have strong sequence similarity around the RGD binding site. Crystal structures of αvβ3 and αvβ3 complexes with RGD ligands have been reported and they reveal an identical arrangement of α5β3 for this interaction [24, 10]. In any case, the basic strategy for RGD recognition, in which the extended tripeptide is held by a two-point anchor located at the well-shaped binding pocket at the subunit interface, seems to be shared among all integrins, as suggested by Takagi [48].

An interesting example is discovered natural mechanism which serves for the enhancement of the fibronectin binding and consists in modifications of non-RGD-bearing sites of the molecule. The modification leads to the non-RGD-bearing sites adopt the right conformation and acquire an ability to be bound in the RGD-binding pocket of integrin receptors. The mechanism is based on the spontaneous transformation from NGR to isoDGR through Asn deamidation in fibronectin modules FN-I5, FN-I11, FN-I17 and FN-II19 driven by the protein-LisoAsp-O-methyltransferase (PMT). It has been well established that most likely this type of the events lead to activation of a latent integrin-binding sites [49, 50]. NMR structure analysis of cyclic isoDGR-2C and RGD-2C peptides together with αvβ3-integrin molecular docking show that isoDGR fits into the RGD-binding pocket in an inverted orientation as opposed to RGD and favorably interacts with this integrin [49, 51]. However, it is known that integrin receptors are capable to distinguish different ligands. For example, receptor αvβ3 binds more preferably with vitronectin molecule than with fibronectin. Detailed investigations of the spatial organization of the RGD-bearing regions of different integrin ligands such as fibronectin, vitronectin, different virus envelope proteins uncover a general rule. Is that RGD-motif is usually positioned in the flexible loop, which may be of varying length and conformation, but always enables the correct conformation of the RGD-motif (β-turn) that is needed to bind to the receptor. At the same time, despite the lack of rigid structure of RGD-flanking regions, it cannot be excluded that the amino acid residues constituting RGD-containing loop may as well interact with the ligand-binding region of the receptor and thus affect the specificity to a particular type of integrins. In other words, it is possible that the selectivity is provided by amino acids flanking the RGD tripeptide.

### 3. AMINO ACID SEQUENCES SURROUNDING RGD-MOTIF IN NATURAL LIGANDS

A number of studies demonstrated that the amino acid residues flanking the RGD-motif of RGD-bearing proteins affect their binding specificity and affinity to integrins [52-57]. Indeed, natural RGD-bearing proteins have different amino acid sequences surrounding RGD-motif (Table 1). However, some studies are demonstrating that by means of substitutions of amino acids in RGD surrounding regions the changes in selectivity relative to an active receptor might be obtained. A wide variety of data has been provided by investigations of, for example, disintegrins and viral proteins.

Disintegrins are small peptide integrin antagonists originally derived from snake venom [58]. Many contain RGD-motifs and act as antagonists of the RGD-recognizing integrins. For instance, kontaktorstatin is known to bind αvβ3, αvβ3, and α5β1 integrins (binding Kd: αvβ3 6.6 nM, αvβ5 19.5 nM, α5β1 191.3 nM [59]). At the present time well known that kontaktorstatin may cause decrease in growth of both hormone dependent and independent tumors in vivo by itself and in combination with conventional cytotoxic [60] and retard cell migration in vitro [61]. Rhodostomin, trilavim and trigramin have been shown to inhibit αvβ3-mediated tumor cell induced platelet aggregation [62], αvβ3-mediated adhesion to the extracellular matrix and tumor growth [63], representing a potential treatment for bone metastasis. These molecules are known to have differences in amino acids flanking RGD, which correlates with binding affinity to different integrins. For example, the presence of an RGDW sequence correlates with a high affinity of disintegrin to the integrin αvβ3, whereas presence of an RGDNP sequence causes the preferential binding to αvβ3 and α5β1 integrins [56]. Analysis of the disintegrins with point amino acid mutations in the RGD flanking region disclosed that the absence of proline residue front of R from RGD sequence is important for effective binding to integrin α5β1. It was also shown that in case of eleganintin in which sequence of RGD motif is ARGDNP, replacement of the N-terminal alanine with the proline decrease its binding to integrin α5β1 [57]. Whereas in case of rhodostomin, having a sequence PRGDMP, substitution of the N-terminal proline to alanine or glycine increase its binding to integrin α5β1 [64]. Most likely the replacement of proline with alanine adjacent to RGD motif in rodomostine affects a dynamics of the RGD-bearing loop. Increase in fast motion on the ps-ns timescale may result in increase of the flexibility and facilitation of exposure of RGD-loop into solvent. This means that the structure namely of the RGD-bearing loop may be responsible for fast recognition and fitting of rodomostine to integrin α5β1 [64-68]. This is consistent with the dynamic features of the RGD-motif in fibronectin, which preferentially binds to integrin α5β1 [69].

Another report demonstrated that the foot-and-mouth disease virus (FMDV) utilizes integrin αvβ6 for the attachment and entry into the host cell [70]. The interaction with αvβ6 is mediated by RGD-motif located at the top of the flexible loop of FMDV VP1. Cultivation of the FMDV in the medium containing the inhibitor of interaction with integrin αvβ6 - bovine secreted soluble αvβ6 (ssαvβ6) - leads to the formation of different strains of viruses
showing moderate to high resistance to the ssαvβ6 [70]. It is notable that such strains retained capability of high affinity binding to host αvβ6. It was shown that abovementioned resistance comes from the substitution of the amino acids in the RGD-bearing region of VP1. The mutations were detected in the RGD-motif and RGD-surrounding amino acids. It should be noted that in VP1 from viruses demonstrating high-resistance to ssαvβ6, namely RGD sequence was mutated via G to D substitution. In VP1 from viruses with moderate resistance to ssαvβ6, substitutions of the amino acids in RGD-motif surrounding have been occurred [70]. Thus, the mutation in RGD-motif leads to formation of the proteins capable of selective binding to the host αvβ6 while showing no binding activity to ssαvβ6. Otherwise, the replacement of the RGD flanking amino acids is not significant to influence selectivity. The data obtained allows for the conclusion that amino acids surrounding RGD motif in VP1 are important for VP1 selective binding to integrin αvβ6.

Our working hypothesis is that in this case RGD serves as a strong high affinity “velcro” whereas the environment - provides the ability to fine-tune the selectivity to certain receptor. Without RGD -probably less effective binding would still be through the other sites but only to a certain receptor (Fig. 1).

Another observation reveals that the presence of the DLXXL-motif in the ligand is important for binding to integrin αvβ6 [71]. It was confirmed by sequence analysis of the proteins capable of selective binding to αvβ6 (namely LAP-1 and VP1 of different serotypes of FMDV). All these polypeptide sequences contain highly conservative sequence RGDXXI (LAP-1) [72] or RGDXXL (VP1 of different FMDV serotypes) [73]. Next, it would be interesting to compare the ligands of closely related integrins α5β1 and αvβ1. These heterodimers differ from each other by α-subunit and αv and αvβ1 preferentially bind peptides, which contain RGDS/TR sequences [18, 74]. RGDS/TR-motif is also common in the ligands of αvβ3 and αvβ5 integrins [18].

Structural analysis of the ligand-recognition domains of integrin receptors has allowed establishing the features providing selectivity with respect to ligands bearing certain amino acid residues in the immediate vicinity to RGD-motif. It was shown that both α and β chains of integrin receptors participate in the formation of RGD-binding site and that the amino acids forming the hydrogen bonds with the side chains of the amino acids of the RGD sequence are similar in different integrin receptors [75].

As for α-subunit: in an attempt to understand the causes of different affinity for different ligands, integrins α5β1 and αvβ1 was compared. Despite the fact that these heterodimers differ only in α-subunit they show a substantial difference in the ability to bind RGD-containing ligands. In particular, α5β1 requires presence of GW C-terminal to RGD for effective interaction with fibronectin while this is not essential for αvβ1. With the aim of mapping of ligand-recognition regions on the αv subunit the set of modified αv proteins was obtained where certain regions, presumably involved in ligand recognition, have been replaced with analogous regions of αv. The result was a fusion protein capable of binding ligands specific for αv, and not capable of binding ligands specific to α5. [75]. The region 107-226 corresponding approximately to repeats 2 and 3 of α5 is sufficient to change all the ligand binding properties of αvβ1 to those of α5β1 as well as replacement of 6 amino acid fragment (Asp154-Ala159) in predicted loop region of αv with the corresponding region of α5 is sufficient to confer selectivity for RGDGW. There is a model in which in a structure of α5-chain (similarly to αvβ) there is a hydrophobic pocket formed by groups of Trp157 and Phe187 (in αvβ - Phe160 and Tyr190) [10]. Thus there exists a possibility for stacking interactions with aromatic groups - phenyl group of phenylalanine in RGDGF and the indole ring of tryptophan in RGDGW peptides [75]. In addition, the peptides containing the RGDW or RGDF sequences are known to be perfect inhibitors of binding of αβ integrin to natural ligands, but at the same time, poor inhibitors of α5β1. In this scenario most likely good inhibitors are peptides containing RGDGW or RGDF sequences [18, 76]. The difference is in the "density" of the bend, which occurs in the polypeptide chain after aspartate. In the case of RGDGW/F it represents a more open structure (less tight) than the RGDW/F due to the presence of glycine after aspartate. Instead of amino acid residues carrying large side groups - Tyr166, Arg214 and Arg216, which form the ligand-binding region of β3, in case of β1 subunit - side chain groups of Ser77, Gly223 and Leu225 are involved in ligand-binding region formation [77, 78]. As a result, the ligand-binding region B1 is more open and is capable of binding more extended turn encountered in the peptides RGDGW/F (Fig. 2).

4. AUXILIARY/SYNERGY SITES IN NATURAL LIGANDS OF INTEGRIN RECEPTORS

RGD flanking regions, as discussed in previous part of the review indicates the structural elements, located at a substantial distance from the RGD-motif, but still capable of binding to integrin receptors and to provide selectivity of natural ligands to target receptor that have been identified. Initially distant site called “auxiliary/synergy” site that was found in the fibronectin molecule and
has a sequence PHSRN. All of RGD-recognizing integrins are able to bind fibronectin. The presence of synergy site leads to enhanced binding of RGD-loop of fibronectin to integrins α5β1 and αIIbβ3 specifically by over forty-fold [79] and does not affect the binding of RGD-motif to, for example, integrin αvβ3. Peptide motif PHSRN is located in the 9th type III domain of fibronectin. Presumably, this site provides preferential binding to integrin α5β1 among other RGD-recognizing integrins [80-82]. Wherein the distance between the RGD-motif and synergy site in fibronectin molecule has been found to be significant and should be about 32 Å. Reduction of the distance between the RGD loop and synergy site (e.g. deletion of only two amino acid residues) greatly reduces the binding ability of fibronectin to integrin α5β1 [79]. A fibronectin mimetic peptide containing RGDSP and PHSRN sequences has

Fig. (1). Schematic drawing the mechanism of the generation of FMDV strains shows the presence of strong and moderate resistance to ssαvβ6. VP1 of the wtFMDV that capable to non-selective binding to both αvβ6 and ssαvβ6. Mutations in the RGD sequence (black circles) lead to emergence of high-selective VP1 capable to recognize αvβ6 but unable to bind to ssαvβ6. Mutations in RGD surrounding (grey circles) lead to genesis of low-selective VP1 with slight preference to αvβ6.

Fig. (2). The positions of the amino acid residues which were demonstrated to be crucial for the recognizing by certain integrins. X - Indicates any amino acid.
been developed to deliver liposomes [83] and/or polymer vesicles [84] to α5β1-expressing prostate cancer cells. Moreover, the PHSRN sequence directed the peptide specifically to α5β1 and increased efficiency of internalization and drug delivery to LNCaP cells compared to GRGDSP. α5β1-targeting of polymer vesicles containing TNF-α increased cytotoxicity up to four times higher compared to free TNF-α [84]. However, in vivo applications of this strategy have not yet been taken into the account. More recently, it was found that highly selective binding of tenasin to α8β1 integrin requires the existence of synergy site located C-terminal to RGD-motif. A series of deletion mutants obtained allowed identifying sequence of this site as LFEIFEIER, wherein crucial motive is EIE. RGD-peptide poorly inhibited neuronectin binding to α8β1 while LFEIFEIER peptide did not inhibit binding at all. Recombinant peptide containing both motifs was shown to be potent inhibitor of tenasin binding to α8β1 integrin - it was about 2000-fold more potent than a peptide containing only the RGD [85]. An auxiliary epitope providing the very high binding affinity to αvβ3 was found at the C-terminus of echistatin [86] and has been proposed for vitronectin [88]. Finally, the αvβ5 integrin may also have an auxiliary binding site in vitronectin which contains highly basic sequence and appears to account for the binding of this integrin to the Tat protein [87].

CONCLUSION

Properly structured RGD-motif is the basis to address to RGD-binding integrin receptors. Since it’s able to provide high affinity of such interaction. Therefore, when it is necessary to bind cell “stronger”, in molecules of extracellular matrix appears maximum number of RGD by means of unmasking of what was "hidden", partial unfolding of the molecule or by modifying sequence generally not containing RGD – e.g., by conversion of NRG in isoDGR.

But what if not only high affinity but also specificity to a particular receptor is needed?

Here the study of the natural ligands suggests at least two more possibilities. Namely certain amino acids in RGD-flanking regions and distant auxiliary/synergy sites, such as synergy site important for binding to α5β1 in case of fibronectin or to αvβ3 in case of echistatin.

In other words, can be said that RGD per se serves as a strong high affinity “velcro” whereas the nearest environment and auxiliary/synergy sites - provides the ability to fine-tune the selectivity to certain receptor. It is mechanism in which the combination of a core interaction (RGD site) with a secondary interaction (e.g., synergy site) ensures both high affinity and specificity. This idea is illustrated in Fig. 3. Undoubtedly, these principles should be able to open new and more effective strategies in the context of the anti-cancer drug design and development of integrin-addressing molecules for the future clinical applications.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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![Fig. (3). The basic strategy for the development of highly selective anticancer agents addressing RGD-binding integrins. Short RGD-peptides (such as cyclic RGD, cRGD) capable of high-affinity binding to RGD-recognizing site of integrin receptors demonstrate low selectivity. The elongation of the peptide by the attachment of the RGD-surrounding leads to increase in the selectivity from low to moderate level. Molecule capable of binding to three specific sites demonstrates the highest selectivity. Integrin X, Y and Z schematically represent ectodomains of three different integrin receptors. A - Auxiliary site. R - RGD-recognizing site. S - surrounding-recognizing site. !!! - Steric hindrances.](image-url)
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