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Review

Structure and function of RGD peptides involved in bone biology

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Abstract. This review focuses on recent papers that describe the involvement of the RGD sequence in bone biology and incorporate the use of synthetic RGD peptides to develop new drugs or control the bioactivity of materials used for bone regeneration. Because in vivo bone function is completely dependent on angiogenesis and vessels, the present publication is focused on physiology, pathophysiology and therapeutics of RGD peptides dedicated to bone cells and endothelial systems. It appears that $\alpha_V\beta3$, $\alpha_V\beta5$ and $\alpha_{IIb}\beta3$ are the integrins most reported to be involved in bone function and RGD sequence binding. The specificity of RGD peptides depends on backbone conformation, orientations of the charged side chains of Arg and Asp residues, and hydrophobic moieties flanking the Asp residue. Despite of recent progress in integrins and RGD peptide structures and function, future work should focus on integrin selectivity of RGD-based agents, model structure and activity-selectivity relationships.

Key words. RGD peptides; bone; osteoblast; osteoclast; endothelium.

Introduction

The ability of cells to adhere to extracellular matrix (ECM) or substratum is an early effect of tissue genesis before the cells proliferate and organize ECM molecules into a functional tissue. Cell adhesion comprises a cascade of four steps: cell attachment, cell spreading, organization of cytoskeleton and formation of focal contacts. ECM molecules that may affect cell adhesion include collagen, thrombospondin, osteopontin, bone sialoprotein I, fibronectin, vitronectin and still-to-be-identified molecules. Fibronectin and vitronectin are believed to be the primary glycoproteins in serum that promote adhesion of various cell types, including bone cells. A key finding remains that the amino acid sequence Arg-Gly-Asp (RGD) in fibronectin serves as a primary cell attachment cue. The RGD sequence is expressed in many ECM molecules, and the responsiveness of the RGD sequence for cell attachment has been shown in many cases. This has been extensively documented in the international literature since Pierschbacher and Ruoslahti laid the foundation in the mid-1980s [1, 2]. A few years later scientists began to investigate preparation and efficiency of synthetic RGD peptides for biomedical purposes.

It appeared that small synthetic peptides (a few hundred daltons) that contain the amino acid sequence RGD can mediate cell attachment similarly its considerably larger parental molecule (a hundred thousand daltons). According to Craig et al. (1995) the potential exists to develop RGD-based therapeutics that function either as agonists to promote the interaction of cells and tissues with artificial matrices, or as antagonists to control the nature of cell-cell and cell-ECM interactions [3]. At this state of the art many different RGD peptides have been developed. Linear and cyclic RGD peptides, and chemically designed peptidomimetics are currently being tested by researchers.

This review focuses mainly on recent papers (i.e. published during early 1980 through early 2002) that describe

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the involvement of the RGD sequence in bone biology and incorporate the use of synthetic RGD peptides to develop new drugs or control the bioactivity of materials in bone metabolism. In vivo bone function is completely dependent on angiogenesis and vessels; the authors propose to focus this presentation on physiology, and pathophysiology and therapeutics of RGD peptides dedicated to bone cells and endothelial systems.

RGD peptide classification

Integrin superfamily

Integrins are a family of cell-adhesion receptors which interact with the ECM and the cytosolic components. Integrins transfer signals bidirectionaly via so-called inside-out and outside-in signalling. They are heterodimeric glycoproteins, consisting of an α and a β subunit. The schematic signalling pathway is activated by binding a ligand, clustering of integrins and cytoskeletal and lysosomal activation, shown in figure 1 [4]. This RGD-binding site is involved in cell morphology, differentiation, proliferation and gene expression. Integrin signalling pathways are although responsible for the survival of the cell, and apoptosis seems to be linked to a disturbance of integrin function. Many integrins express RGD-dependent binding, e.g. $\alpha_V\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_6$. Beneath the RGD binding site exists a heparin binding site which is partially involved in ligand binding. How the two binding sites react together is not yet clear [5, 6]. The proteins that interact most with RGD-dependent integrins are fibronectin, vitronectin, bone sialoprotein, osteopontin, thrombospondin, fibrinogen, laminin, collagen, nectipepsin and other unknown molecules [7, 8]. A very interesting fact is the use of the RGD sequence by pathogenic microbes. Bacteria, viruses and yeasts use the RGD sequence to get into the host cell [9, 10]. Humphries et al. (1995) explored the RGD competitive sequence QAGDV and LDV in integrin binding [11]. The integrin subunits have been identified on different type of cells. The integrins reported most commonly in the literature are $\alpha_V\beta_1$, $\alpha_2\beta_1$, $\alpha_1\beta_1$, and $\alpha_V\beta_3$, as shown in table 1.

Integrin-binding peptide sequence and classification

According to the above classification, integrins are selective toward their substratum ligands. The specificity is a central issue in the use of cell adhesion peptides and surface engineering. Whether peptide sequences that promote cell adhesion will in the future exhibit specificity for a single cell type remains unclear. Integrins showed different binding activity for RGD peptides. If one specific cell

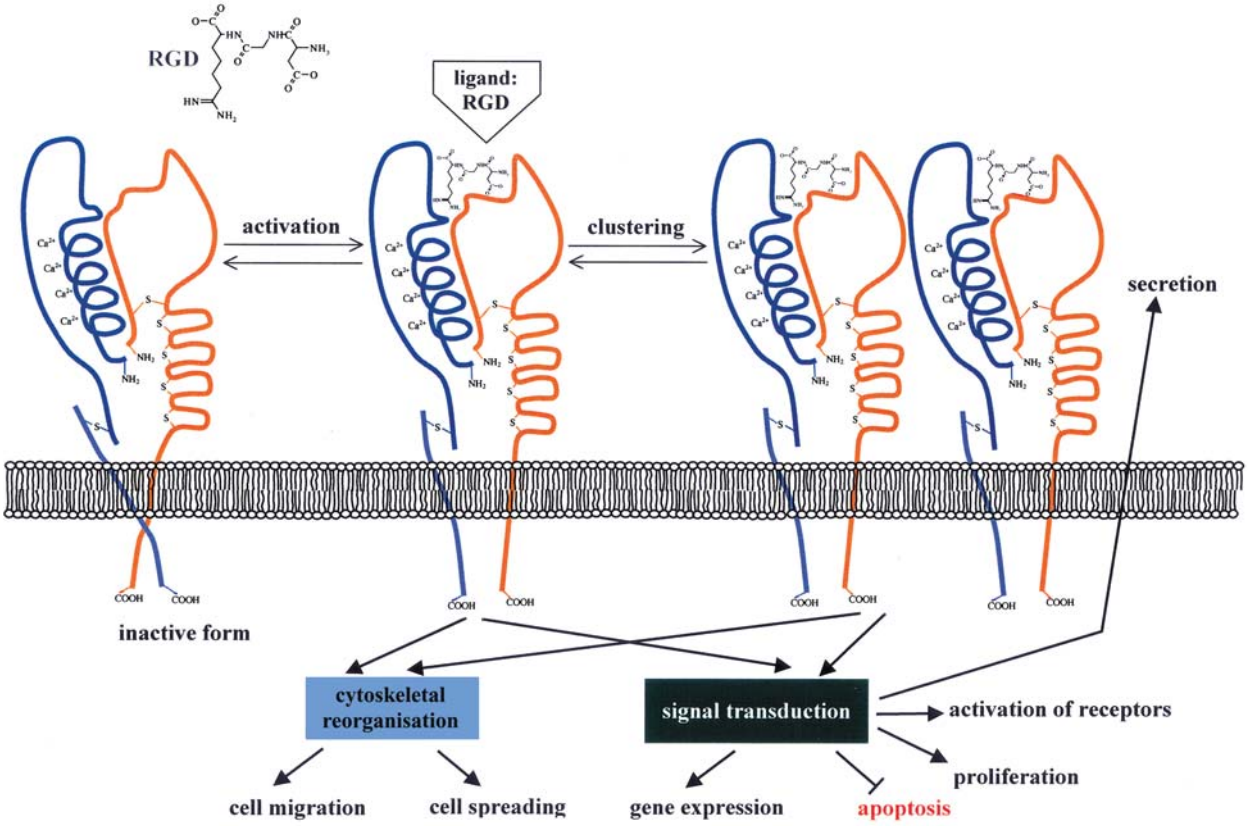

Figure 1. Scheme of binding the RGD peptide to an integrin. Influence of RGD-peptide binding and possible ways into the cell.

Table 1. Integrins and ligands.

Subunits		RGD dependence	Ligands	Cell type
β_1	α_1	+	LM, Col IV+I	LC, SM, ML, OB
	α_2	– (+)	LM, Col, FN	EC, LC, MC, NP, TC, OC, OB
	α_3	+/–	FN, LM, Col I,epiligrin	EC, LC, MC, ML, OB
	α_4	–	FN, V-CAM	LC, MC, OB
	α_5	+	FN, L1	EC, LC, MC, TC, OB
	α_6	–/+	LM	EC, LC, MC, TC
	α_7		LM	TB, MU, ME
	α_8		FN, VN, TN	
	α_9		TN	EP
	α_V	+	VN, FN, FG, L1, Col, LM, OP	ME, CA, ML, FB, OC, SM
β_2	α_L	–	ICAM-1, ICAM-2	LC, MC, NP
	α_M	+?	C3bi, factorX, FG, ICAM-1 β-glucan, leishmania gp63	MC, NP
	α_X	?	FG, C3bi, β-glukan	MC, NP
β_3	α_{IIb}	+	FG, Fx, VN, FN, vWF, TS, fibrin, L1	MK, TC
	α_V	+	VN, FG, FN, vWF, TS, OP, BSP, PB, Col, L1, TN	EC, LC, MC, NP, TC, SM, OC, FB, OB
β_4	α_6	+/–	LM(?)	EP
β_5	α_V	+	VN, vWF, FN, BSP, OP	OC, OB, SM
β_6	α_V	+	FN, VN	EC
$\beta_7(=\beta_p)$	α_4		FN, V-CAM, Md-CAM	
	α_5	–	Peyers patch, addressin, FN, VCAM-1	FB, LC
	α_{IEL}			
β_N	α_V		FN, Col	
β_8	α_V	+	VN, FN	

Ligands: BSP, bone sialoprotein; C3bi, inactive type of C3b component of complement; factor X, coagulation factor X; FG, fibrinogen; FN, fibronectin; Fx, fragment x of FG; Col, Collagen; L1,: 6th Ig-similar domain of cell adhesion molecule L1; LM, laminin; OP, osteopontin; PB, penton base of adenovirus; TN, tenascin; TS, thrombospondin; VN, vitronectin; vWF, von Willebrand factor. Cell types: CA, carcinoma; EC, endothelial cells; EP, epithelial cells; FB, fibroblasts; LC, lymphocytes; MC, macrophages; ME, melanoma; MK, megacaryocytes; ML, melanocytes; MU, muscle cells; NP, neutrophil cells; OC, osteoclasts; SM, smooth muscle tissue; TB, cytotrophoblasts; TC, thrombocytes [3, 5, 6, 38, 78, 114–116].

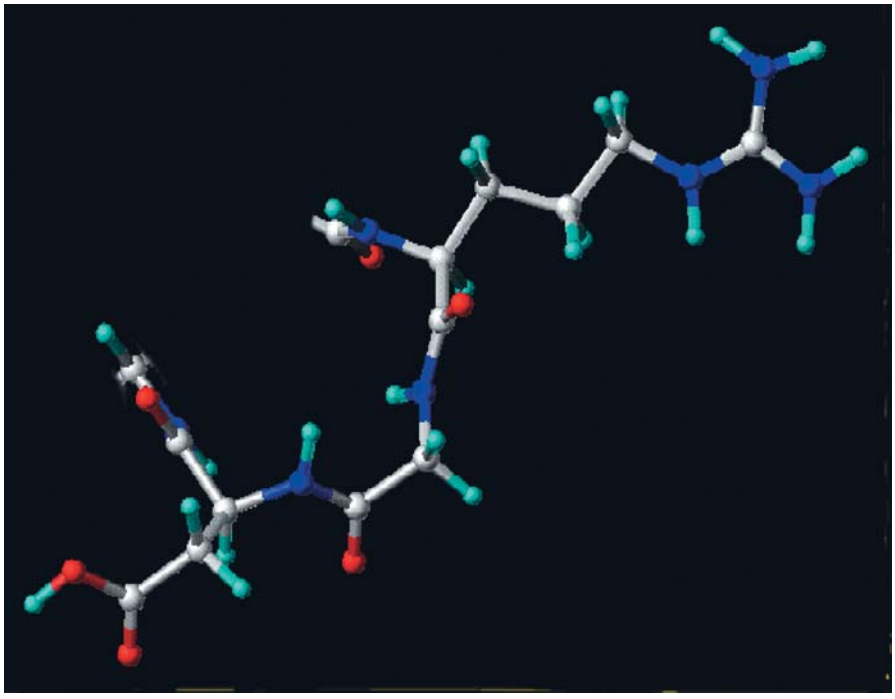

Figure 2. Molecular model of the RGD sequence conformation of the cyclic RGD peptide c(RGDfK) (EMD 121974, listed in table 3). (Reprinted with permission of A. Jonczyk.)

type expresses a distinct integrin pattern, it can only bind to the RGD sequence which is specific for the integrins it expresses. This opens a possibility to control binding of cells to RGD peptides and mediate specific cell attachment to surfaces. Figure 2 shows a molecular model of RGD sequence conformation for the cyclic RGD peptide c(RGDfK) (EMD 121974, table 3).

The affinity of RGD peptides for their ligands may be affected by steric conformation of the peptide [12]. Several peptides that contain RGD serve as ligands for integrins [13]. It has been suggested that substrata composed of RGD not only promote cell attachment but may also enhance other fundamental cell functions (e.g. proliferation) [14]. The amino acid sequences flanking RGD also may affect the selectivity and affinity of peptides toward integrins [15, 16]. For initial cell adhesion, Rezania et al. (1999) proposed the collagen receptor $\alpha_2\beta_1$ for osteoblast-like cells. For spreading, cytoskeletal organization, focal contact formation and possibly migration they proposed $\alpha_V\beta_3$ (vitronectin receptor) [17]. An additional interesting point is the influence of RGD-mediated cell-matrix interactions in bone cell mechanotransduction suggested by Salter et al. (1997) [18].

Chemists synthesize RGD peptide amphiphiles to influence the specific binding of RGD peptides. Synthetic dialkyl lipid tails have been linked to the amino-terminus, carboxyl-terminus and both termini of the cell recognition sequence RGD to produce amino-coupled, carboxyl-coupled and looped RGD peptide amphiphiles. It has been established that amphiphiles are the most effective in influencing human melanoma cell response when the RGD sequence is made accessible through formation of a flexible loop conformation [19]. According to these authors, future studies with new versions of RGD peptide amphiphiles that contain longer RGD sequences, both linear and cyclic, have the potential to further clarify the role of sequence accessibility and conformation in promoting specific cell responses [19].

Nicolaou et al. (1998) describe a group of nonpeptide mimetics based on the structure of the RGD peptides. They tested binding selectivity and ability to inhibit cell adhesion among different integrins ($\alpha_V\beta_3$, $\alpha_V\beta_5$, $\alpha_{IIb}\beta_3$). The tested library showed excellent results for five nonpeptide mimetics generated [20].

Linear RGD peptides

The binding of RGD peptides to different integrins showed a different binding pattern which is influenced by chemical alteration of the side flanking amino acids. Examples of linear peptides and their origin are shown in table 2.

Early studies with RGD peptides demonstrated the influence of stereochemistry and conformation on binding specificity in cell adhesion. In linear peptides, the fourth amino acid alters the binding specificity. Hern et al. (1998) showed in their in vitro re-endothelialization studies with YRGDS and GRGDS that the distance of the presented RGD sequence from a surface promotes specific cell adhesion [21]. Cyclization of a peptide showed an increase in activity and a change in integrin specificity. The specificity changed from fibronectin to vitronectin [22, 23]. In linear peptides, the nature of residues flanking the RGD sequence could influence receptor affinity, receptor selectivity and other biological properties [24]. Tetrapeptides RGDS, RGDV, RGDT were synthesized using an improved liquid-phase procedure. It was found that RGDV exhibits remarkable cell-attachment activity when tested on L-929 fibroblasts. The valine residue contributes to RGDX conformation to fit to the structure of receptor on the cell surface [25]. A review by Healy (1999) on biomimetic engineering of materials addressing the RGD peptide problem concludes with the following words: 'It is unlikely that materials modified only with the ubiquitous linear RGD signal will lead to controlled responses of a specific cell type in complex environments (e.g. in vivo)' [26]. This limitation is one of the reasons for developing cyclic RGD peptides.

Cyclic RGD peptides

In cyclic peptides the RGD peptide sequence is flanked by other amino acids to build a ring system. These systems offer the possibility to present the RGD sequence in a specific conformation. Cyclic peptides are much more potent and more specific than their linear counterparts, and their advantage is their resistance against proteolysis. Table 3 shows different cyclic peptides and their origin.

Cyclic RGD peptides have been developed for various purposes: fibrinogen receptor antagonists [27], selective $\alpha_V\beta_3$ integrin antagonists for treatments of human tumor metastasis and tumor-induced angiogenesis, phagocytosis of cells undergoing apoptosis, bone remodelling and osteoporosis, diabetic retinopathy and acute renal failure. Stereochemistry influence in c(RGDFV) of used D-amino acids was shown by Müller et al. (1992). The use of D-Phe raises the IC50 (the concentration required to inhibit 50% of the tumor cells) on tumor cells 1000-fold [28]. Each backbone amide bond of (RGDfV) was successively *N*-methylated to result in a series of five monomethylated cyclic pentapeptides. The authors propose five classes of cyclic peptides and their representative RGD arrangements with different integrin binding specificities. The influence of this *N*-methylation scan on biological activity revealed thate (RGDf-N(Me)V) is one of the most active and selective $\alpha_v\beta_3$ integrin antagonists known [12].

Stereoisomerism has a great influence on biological activity of c(RGDfV). Studies on retro-inverso peptides showed that the backbone conformation, the side-chain topology of the peptides and the amide bond direction

Table 2. Linear peptides.

Linear peptides	Integrins/proteins		Reference
GRGDSY GRGDVY GRGESY = negative control	$\alpha_V\beta_3/\alpha_V\beta_3$	VN	Lin et al. 1994 [117]
RGDC	vasoactive peptide		Xia et al. 1997 [118]
BRB10 BRB9 BA3 = EPRGDNYRG	$\alpha_V\beta_3/\alpha_V\beta_5$	BSP	Sung et al. 1998 [119]
EPRGDNYR EPRGENYR = negative control	$\alpha_V\beta_3/\alpha_V\beta_5$	BSP	Sasaguri et al. 2000; v. d. Pluijm et al. 1994 [85, 120]
RGDS RGDV Ac-RGDV-NH_2 Ac-RGDS-NH_2 GRGESP = negative control GRGES = negative control GRGDdSP = negative control Ac-(CIT)GDS-NH_2 = negative control	$\alpha_V\beta_3/\alpha_V\beta_5$	VN, FG	Horton et al. 1993 [79]
GdRGDSP GRGDTP GRGDSP GRGDS GRADSP= negative control	$\alpha_V\beta_3/\alpha_V\beta_5$	VN, FG	Horton et al. 1993; v. d. Pluijm et al. 1994; Kumar et al. 1994; Sung et al. 1998 [79, 119, 121]
VTYAVTGRGDSPASSKP TNIMEILRGDFSSANNR TSSTSYNRGDSTFESKS ECKPQVTRGDVFTMPED VVTGSPERGDQSSWKSV TVDTYDGRGDSVVYGLR	$\alpha_V\beta_3/\alpha_V\beta_5$	FN, FG, FG VN vWF OP	Humphries et al. 1995 [11]
G(Pen)GHRGDLRCA Pen substitutes R(Pmc) GHRGDLRCA Substitutes at position 3, –1, –2, combination of positions 1 and 3, = 48 different peptides!	$\alpha_{IIb}\beta_3$		Cheng et al. 1994 [24]
$(GRGDSP)_4K$ = repeated linear P	$\alpha_V\beta_3$	FN	Dettin et al. 2002 [100]

Abbreviations, see table 1.

lead to drastically different inhibitory activities to the $\alpha_V\beta_3$ receptor [29]. Structures of the peptide loops alters activity on receptor binding to fibrinogen. Loops were stabilized by building blocks with kanthene, phenoxazine and phenothiazine derivatives [28]. The possibility of disulfide bonds at cysteine residues in the structure of cyclic RGD peptides alters the binding activity of the peptides [30]. Bogdanowich-Knipp et al. (1999) compared the solution stability of a linear and a cyclic RGD peptide as a function of pH and buffer concentration [31]. It appeared that the cyclic peptide is 30-fold more stable than the linear peptide at pH 7. The degradation mechanisms of both peptides primarily involved the aspartic acid residue. This degradation leads to the loss of biological activity [32, 33].

It has been clearly demonstrated that the increase in stability of the cyclic peptide compared with the linear one is due to decreased structural flexibility imposed by the ring [34]. Cyclic RGD peptides react like native cell adhesion molecules, such as fibronectin, in binding specifically to an integrin [35]. This suggests that cyclic RGD peptides may function as more efficient mediators of osteoprogenitor-cell adhesion than linear peptides [16]. Studies of cyclic RGD peptides immobilized on surfaces showed that such conformationally restrained peptides were more potent in promoting cell adhesion than their linear counterparts [36, 37].

Competitive peptides and peptidomimetic integrin antagonists

A family of snake venoms is known as integrin antagonists and defined as disintegrins. The snake venoms block the function of the integrin receptor completely. Different kinds of snake venoms address different integrins. Echis-

Table 3. Cyclic peptides.

Cyclic peptides	Integrins /proteins		Reference
(Pmc)GHRGDLRCR Ac-CIPRGD(Y-OMe)RCNH$_2$ Ac-CNPRGD(Y-OMe)RCNH$_2$	$\alpha_{IIb}\beta_3$	TC	Craig et al. 1995 [3]
Ac-G(dR)GDSPASSK-GGG(dR)LLLLLL(dR)NH$_2$= Peptide 2000 (Telios)[a]	$\alpha_V\beta_3$	FN	Craig et al. 1995 [3]
CBA4 = DPA-EPRGDNYRCYS-NH$_2$	$\alpha_V\beta_3/\alpha_V\beta_5$	BSP	Sung et al. 1998 [119]
BSA-CM-A-DPA-EPRGDNYRCysNH$_2$= BSA-CNB DPA-EPRGDNYRCysNH$_2$= CNB Ac-DPA-EPRGDNYRCysNH$_2$= Ac-CNB EPRGDNYRCysNH$_2$= C-BC3 dYRGDNYRCysNH$_2$= C-CB1	$\alpha_V\beta_3/\alpha_V\beta_5$	BSP	v. d. Pluijm et al. 1996 [127]
G4120 = cycRGD[b]	$\alpha_V\beta_3/\alpha_V\beta_5$	VN	Horton et al. 1995 [63]
CycGPenGRGDSPCA = peptide 2000 (Telios)[a]	$\alpha_V\beta_1$ $\alpha_V\beta_3/\alpha_V\beta_5$	LM VN	v. d. Pluijm et al. 1994; Kumar et al. 1994 [85, 121]
Ac-CGGNGEPRGDTRAY-NH$_2$	$\alpha_V\beta_3/\alpha_V\beta_5$	BSP	Rezania et al. 1999 [17]
Ac-C(NMe)-RGD-PenNH$_2$= SK&F 106760[c] Ac-C(NMe)-RGN-PenNH$_2$= negative control	$\alpha_V\beta_3/\alpha_V\beta_5$	VN	Horton et al. 1993 [79]
XantheneRGDF XantheneARGDFP XantheneIARGDFPD XantheneRIARGDFPDD XantheneARIARGDFPDDR	flavoridin		K. Müller et al. 1992 [28]
C(GRGDSPA) C(GRGD) C(RGDS) C(GRGDSP) C(GRGDS) C(RGDSP) C(RGDSPA)	$\alpha_V\beta_3$	FN	Kumagai et al. 1991 [23]
EMD 121974 = c(RGDfK)[d]	$\alpha_V\beta_3/\alpha_V\beta_5$	VN	Mitjans et al. 2000 [111]
cRGDVGS-BTD-SGVA	$\alpha_V\beta_3/\alpha_V\beta_5$	VN	Tran et al. 1997 [128]
cRGDRGD	$\alpha_V\beta_3/\alpha_V\beta_5$	VN	Burgess et al. 1996 [129]
cPRGD-Mamb[c]	$\alpha_V\beta_3/\alpha_V\beta_5$	VN	Peishoff et al. 1992 [130]
c(-N(Me)R-GDfV) c(R-Sar-DfV) c(RG-N(Me)D-fV) c(RGD-N(Me)f-V) c(RGDf-N(Me)V-) = EMD 121974[d]	$\alpha_V\beta_3/\alpha_V\beta_5$	VN	Dechantsreiter et al. 1999 [12]
c(RGDFV) c(RADFV)	$\alpha_V\beta_3/\alpha_V\beta_5$	VN	G. Müller et al.1992 Gurrath et al. 1992, Verrier et al. 2002 [16, 35, 131]

Abbreviations, see table 1.
[a] Telios Pharmaceuticals, La Jolla, CA, USA; [b] Genentech, South San Francisco, CA, USA; [c] SmithKline Beecham, Collegeville, USA; [d] Merck KGaA, Darmstadt, Germany.

tatin, kistrin and contortrostatin are described as special $\alpha_V\beta_3$ disintegrins [38, 39]. They are developed from snake venoms and directly influence the β_3 subunit of the integrins, blocking their function. Flavoridin belongs to the snake venom family and potently inhibits blood platelet aggregation [40]. Barbourin, a single disintegrin peptide, contains RGD and KGD. KGD derivatives have a high specificity for platelet aggregation factor [41].

Hoekstra and Poulter (1998) developed RGD-type peptidomimetic integrin antagonists against $\alpha_V\beta_3$ and $\alpha_4\beta_1$ [42]. They described the advantages of RGD peptidomimetics from the chemical point of view: building small libraries, fast and easier synthesis, and same or better biological activity than RGD peptides. The RGD peptides are the first generation of antagonist molecules that mimic activities of matrix proteins, and the RGD peptidomimetics are the second generation. Examples for competitive peptides and peptidomimetics are listed in table 4.

Table 4. Competitive peptides.

Competitive peptides and peptidomimetics	Integrins/proteins		Reference
RRETAWA	$\alpha_5\beta_1$		Mould et al. 1998 [122]
LHGPEILDVPST=CS1 YIGSR	$\alpha_V\beta_3$ laminin P1-fragment]	FN	Horton et al. 1993 [79]
KGD	Barbourin		Scarborough et al.1993 [123]
LDV KQAGD / QAGDV DGEA KKQRRR HRNRKGV	$\alpha_V\beta_3$ $\alpha_V\beta_3$ $\alpha_V\beta_3$ viral protein (tat) related sequence	FN FG Col	Dresner-Pollak et al. 1994; Humphries et al. 1995; Kostenuik et al. 1996 [11, 124, 125]
cDFKRG	$\alpha_V\beta_3$		Verrier et al. 2002 [16]
3863 [a]	$\alpha_V\beta_3$		Gourvest et al. 2001 [106]
SC 56631 peptidomimetic [b]	$\alpha_V\beta_3$		Engleman et al. 1997 [105]
SC 68448 peptidomimetic [b]	$\alpha_V\beta_3/\alpha_V\beta_5$	VN	Carron et al. 1998 [112]
L-748,415 peptidomimetic	$\alpha_V\beta_3$		Rodan et al. ASBMR 18th annual meeting [110]
nitroaryl ethers peptidomimetic benzimidazole peptidomimetic	$\alpha_{IIb}\beta_3$		Nicolaou et al. 1998 [20]
SB 223245 peptidomimetic [c]	$\alpha_V\beta_3$ /$\alpha_{IIb}\beta_3$		Keenan et al. 1998 [126]
SB 265123 peptidomimetic [c]	$\alpha_V\beta_3/\alpha_V\beta_5$		Lark et al. 1999 [109]

Abbreviations, see table 1.
[a] Roussel-Uclaf, Romainville, France; [b] Monsato Company, St. Louis, MO, USA; [c] SmithKline Beecham, Collegeville, USA.

Physiology

The receptors of the integrin family are found in many parts of the body. A search through sequence databases of receptor proteins revealed the cell adhesion motif RGD in 67 integral plasma membrane proteins [43]. Inspection of all known protein three-dimensional structures containing an RGD sequence and not having a documented cell adhesion function shows that such a sequence is mostly part of a loop [43]. This finding showed the many different possibilities where RGD peptides could be involved. For our review the role of the RGD is described in the endothelial system and bone, because in the body bone exists only with and because of the endothelial system.

Endothelial systems

The endothelial system is one of the major parts in living bodies. As outlined by several authors [44–46], cell shape plays a critical role in growth control. It appears that cell spreading turns off differentiation, whereas cell retraction or rounding maintains differentiation-specific functions and prevents cell proliferation. This has been clearly demonstrated for capillary endothelial cells, where cell shape exerts distinct regulatory signals that are responsible for switching cells between growth and differentiation programs [47]. Binding to transmembrane integrin receptors and thereby activating both chemical and mechanical signalling pathways is a key issue during this complex cellular process [48]. Capillary endothelial cell adhesion to ECM and associated integrin binding can directly activate signalling cascades in quiescent endothelial cells that stimulate these cells to reenter the growth cycle [49]. Angiogenesis is an anchorage-dependent process that can be inhibited by interfering with the attachment of endothelial cells to the ECM. Therefore, RGD-containing synthetic peptides can be used to study the mechanisms by which the ECM regulates angiogenesis [50].

The blood vessel growth is controlled, in part, by the matrix surrounding it, in particular, the basement membrane underlying the endothelium [51]. ECM exerts a local control mechanism which modulates cell sensitivity to soluble stimuli (angiogenic growth factors) [52]. Endothelial cells express β_1 ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$) and β_3 ($\alpha_V\beta_3$) integrin group [53–59]. The $\alpha_V\beta_3$ integrin is selectively expressed on endothelial cells that are engaged in angiogenesis [60]. Bayless et al. (2000) present very convincing data showing that the integrins $\alpha_V\beta_3$ and $\alpha_5\beta_1$ regulate human endothelial cell vacuolation and lumen formation in three-dimensional fibrin matrices [61]. This implicates a major role for these two integrins in endothelial cell morphogenesis.

Cyclic RGD and linear RGD peptides both bind to the integrin $\alpha_V\beta_3$ of bovine aortic endothelial cells, but immobilized cyclic RGD peptide exhibited a greater affinity than a linear RGD peptide did [37]. Increasing evidence exists that α_V integrins are upregulated in new capillaries that proliferate in response to angiogenic stimulus [62]. Thus, in certain pathologies (wound repair, association with cancer) the modified phenotype of vascular cells may result in, or be due to, alteration in integrin expression and hence cellular behaviour. There is a possibility that α_V integrins may thus be a useful target for diseases characterized by neovascularization [62].

Bone

The cells that are found in bone system are osteocytes, osteoclasts and osteoblasts. Because osteocyte physiology is only rarely documented, we focus on osteoblasts and osteoclasts (fig. 3). Osteoblasts are the cells that generate new bone, and osteoclasts resorb bone. These two cell types are relevant for the cascade of bone remodelling. The following integrin receptors have been detected in human bone generally: $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_V\beta_3$, $\alpha_V\beta_5$ (table 1, integrins and ligands). The two ECM-bone proteins osteopontin and bone sialoprotein, which are involved in bone resorption and bone building, contain the RGD binding motif. The RGD motif is essential in osteopontin – mediated osteoclast cell binding. Bone sialoprotein was found only in mineralized tissue and not in osteoclasts [63, 64]. Other glycoproteins that are found in bone matrix are thrombospondin, fibronectin and vitronectin [7].

Collagen is one of the major proteins in bone. Around 95% of all bone protein is collagen I. Distinct collagen subtypes are recognized by specific cell surface receptors. Two collagen receptors, $\alpha_1\beta_1$ and $\alpha_2\beta_1$, are members of the integrin family [65]. It has been established that the $\alpha_2\beta_1$ integrin binds to a site within the α1(I)-CB3 fragment of type I collagen [66]. Rezania and Healy (1999) demonstrated that initial attachment (first 30 min) of human os-

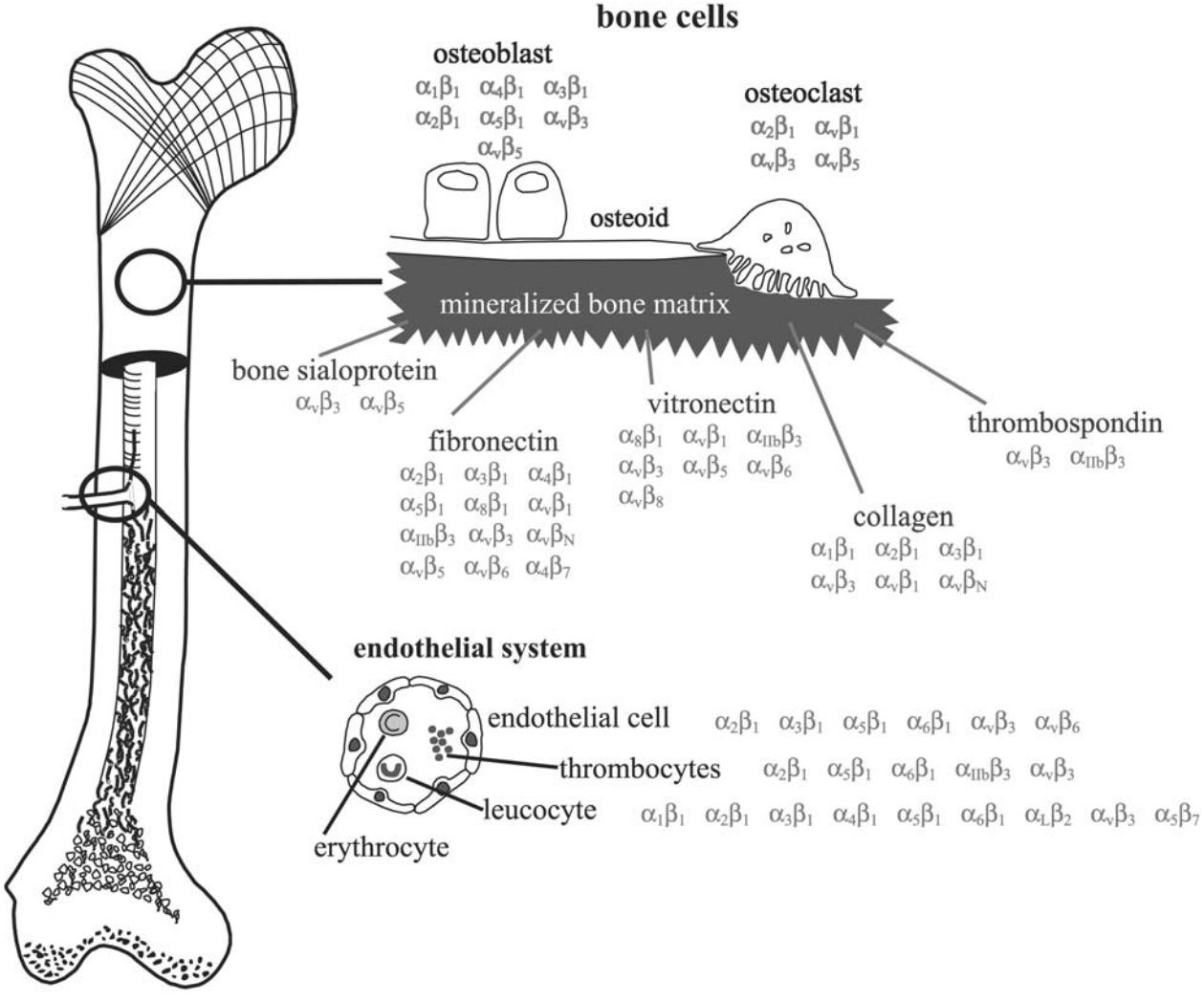

Figure 3. Depiction of bone structure in general and bone cells, endothelial system and involved integrins.

teoblast-like cells to homogenous RGD surfaces was mediated by the collagen receptor $\alpha_2\beta_1$, whereas the receptor $\alpha_V\beta_3$ governed longer-term adhesion [67].

Osteoblast

The integrin receptors $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_V\beta_3$ have been detected in osteoblasts obtained from rat calvariae. According to stage of differentiation and environmental conditions (in vitro, in vivo), the expression of integrin receptors may be different [68]. Although cultured osteoblasts were found to express the integrin $\alpha_V\beta_5$ [69], this receptor was not detected on osteoblasts in human tissue [70]. This is due to the fact that cell lines adopt living modifications other than their primary counterparts.

Studies have established that heterogeneous mimetic peptide surfaces containing both the RGD and FHRRIKA (putative heparin-binding) peptides in the ratio of 75:25 or 50:50 proved to be biologically relevant for rat calvaria osteoblast cell function (the RGD signal is required for promoting formation of focal contacts and cytoskeletal organization) [71]. RGD sequence isolated from bone sialoprotein enhanced osteoblast cell attachment and promoted cell spreading and focal contact formation [17, 68]. The RGD fragment by itself regulates mineralization; this process is not dependent on confluence of cells [17, 72]. Dee et al. (1997) proposed that the RGD sequence alone is not sufficient for optimal cell colonization. For a well-designed biomaterials surface they recommend an integrin and a proteoglycan-mediated prepared surface [73].

Osteoclast

Osteoclasts express the integrin subunits α_V, α_2, α_5, β_1 and β_3, and the integrin receptors $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$ and $\alpha_V\beta_5$ [74, 75]. The integrin $\alpha_V\beta_3$ was mainly described for osteoclast adhesion and migration in correlation with expression of osteopontin as the main ligand for osteoclasts in vivo [75].

Echistatin and different RGD peptides blocked the increase of intracellular Ca^{2+}, which was immobilized by ligand binding to vitronectin receptor [76, 77]. This implicates an RGD-dependent adhesion mechanism. Horton et al. (1993) tested many linear and cyclic peptides on seeded osteoclasts [79]. The linear peptide GRGDS competed with osteopontin-dependent osteoclast cell binding, whereas GRGES has no effect on osteoclast attachment. The osteoclasts showed a retracted cell shape in a dose-dependent manner. The cyclic peptides showed a 10-fold higher activity in osteoclast retraction than their linear pendant. The most potent osteoclast retraction substance was the disintegrin echistatin [79]. Echistatin was also found to induce osteoclast retraction as well as to reduce pit formation in bone slices [80, 81]. Nakamura et al. (1998) described the inhibition of osteoclast as well as osteoclast precursor migration and formation of multinucleated cells in vitro by echistatin in an osteoblast coculture system [82]. Flores et al. (1992) showed the influence of bone sialoprotein in bone resorption and osteoclast formation [82]. Inhibition of osteoclast adhesion to bone surfaces, osteoclast retraction and inhibition of bone resorption by RGD peptides, RGD-containing peptides or antibodies to $\alpha_V\beta_3$ are described in the literature [81–85]. Competitive antibody studies show $\alpha_V\beta_3$ to be the predominant receptor in this case [86]. An animal model with thyroparathyroidectomized (TPTX) rats treated with echistatin presented by Fisher et al. (1993) showed for the first time that the RGD sequence was required for inhibition of bone resorption in vivo [87]. These results were confirmed by King et al. (1994), with another disintegrin, kristin. Contortrostatin completely inhibited osteoclast attachment to vitronectin and fetal bovine serum [39].

Pathophysiology and therapeutics

Despite the differences among the models proposed, it is agreed that the following factors are important for biological activity of RGD peptides: (i) the backbone conformation of the RGD sequence, (ii) the orientations of the charged side chains of Arg and Asp residues and (iii) the hydrophobic moiety flanking the Asp residue [88]. Limited information about integrin selectivity of RGD-based agents has been reported, and very little is known about the actual structure and activity-selectivity relationship [3]. Tissue regeneration is a target for therapies using agonist RGD-based compounds [89]. The diseases targeted by integrin antagonists are based on ECM dysfunction. The diseases are highly diverse and include cardiovascular disease, cancer, osteoporosis and inflammation. In addition, conditions such as burns, organ and tissue regeneration, and chronic wounds are targets for therapies using agonist RGD-based compounds [3]. RGD peptides are even used in the field of tissue and organ engineering. Jeschke et al. (2002) are working on generating artificial articular cartilage with cyclic RGD peptides [89a].

Endothelial systems

Fibrinogen receptor antagonists are designed with RGD peptides to work orally as antithrombotic therapy agents [90, 91]. Because the integrin $\alpha_V\beta_3$ is expressed on endothelium and tumors, it may contribute to a malignant phenotype by supporting the growth and persistence of small blood vessels that nourish primary and metastatic tumors. Thus RGD peptides or peptidomimetics or anti-integrin antibodies designed as ligands for $\alpha_V\beta_3$ can inhibit tumor growth and development by blocking tumor-induced angiogenesis [12, 92, 93].

Binding of synthetic RGD peptides with specific ECM receptors (e.g. $\alpha_V\beta_3$) on the endothelial cell surface induces

a capillary regression or involution, including apoptosis [94]. Furthermore, it has been shown by Mogford et al. (1997) that interaction of RGD peptides with endothelial cell $\alpha_5\beta_1$ integrins causes arteriolar vasoconstriction and may play an important role in vascular control [95]. In the field of bone engineering the need for endothelial cell cultures in three-dimensional models and coculture with bone cells in biological scaffolds must be stressed [96].

Bone

Application of adhesion peptides in conjunction with material surfaces has become an active and important area of research in the field of cell and tissue engineering. The materials that are used in the field of biomaterials and implantation often lead to poor integration of the graft into the surrounding tissue. Graft rejection, low mechanical stability of the biomaterial-tissue interface, infections and inflammations are the side effects. The coating of biomaterials with RGD peptides offers an attractive strategy for controlling the cell-material interface and achieving a bioactive implant [16, 97]. The advantage of synthetically prepared peptides is precise control of their chemical composition and their favourable immobilization on an inert surface. Relative to high molecular weight proteins, peptides are more resistant to denaturing insults such as variations in pH and heat. Healy et al. (1992) propose a convincing strategy to design biomimetic materials to direct biological responses [98]. RGD peptides as a drug are under investigation for treatment of osteoporosis, mainly based on their activity on osteoclasts.

Osteoblast

Osteoblasts are responsible for generating mineralized bone tissue. It is important for bone neogenesis that osteoblasts generate a stable cell surface. Many groups are working on modifying surfaces. Rezania and Healy (1999) demonstrated that utilizing peptide sequences incorporating both cell- (RGD) and heparin-adhesive (FHRRIKA) motifs can enhance the degree of cell surface interactions, proliferation and influence the long-term formation of mineralized ECM by rat calvaria osteoblast-like cells in vitro [17].

Sofia et al. (2001) functionalized silk surfaces with RGD peptides and parathyroid hormone. They cultured osteoblast-like cells on functionalized surfaces and showed a 25% increase in differentiation studies for RGD-modified silk [99]. An approach for dental application was described by Dettin et al. (2002). To select the best RGD sequence presentation on polystyrene, they designed branched or repeated RGD peptides. A linear peptide in which the sequence GRGDSP [$(GRGDSP)_4K$] was repeated four times enhanced osteoblast and endothelial cell adhesion [100]. Surface modification of polylactid films and structures with linear RGD peptides support bone marrow cell growth, differentiation and mineralization [101]. A healing wound model provided information about the positive influence of osteocompatibility and the negative inflammatory potential of a linear RGD peptide [102]. Our group developed a system to functionalize polymethylemethacrylate with cyclic RGD peptides. In vitro evaluation showed enhanced osteoblast adhesion, but no influence on differentiation. The animal studie results in direct in- and ongrowth of bone by coated implants and a fibrous layer formation for uncoated implants [103].

Osteoclast

Osteoclasts play a key role in bone loss in osteoporosis due to their function in bone resorption. RGD peptides are one strategy of preventing bone loss in this disease. The models proposed here used conventional therapeutics such as oestrogen and alendronate or RGD sequence-containing disintegrins, for example echistatin, contortrostatin and kistrin, and peptidomimetics.

Experiments with ovariectomized mice and rats showed similar effects in preventing bone loss using the pharmaceutics oestrogen and alendronate [38, 104]. Mercer et al. (1998) showed inhibition of osteoclast attachment by kistrin and contortrostatin in cell experiments [39]. An acute animal model used by Horton et al. (1991) and Fisher et al. (1993) showed inhibition of bone resorption by echistatin and kistrin in vivo [84, 87]. Other in vivo models of bone resorption demonstrated that echistatin and RGD-containing peptides inhibited bone resorption in ovariectomized rodents [38, 104, 105]. These results suggest the potential of RGD peptides as therapeutic agents for osteoporosis. In contrast, Sato et al. (1994) found that synthetic echistatin inhibits bone resorption incompletely. They suggest the involvement of other integrins in attachment, detachment and resorption [80]. Based on the RGD structure, synthetic RGD-mimetic compounds were designed. These mimetic compounds are effective inhibitors of bone resorption in vitro and in vivo [105–108]. An orally active RGD peptidomimetic vitronectin antagonist was described by Lark et al. (1998) to prevent osteoporosis [109]. SB 265123 showed inhibition efficiency in a TPTX rat model for preventing bone resorption. There was minimal influence on platelet aggregation and the risk of bleeding was minimized. This could be the first step of a treatment for postmenopausal osteoporosis. Osteoporosis treatment was proposed by Rodan et al. (2002), who used an $\alpha_v\beta_3$ low molecular peptidomimetic L-748,415 and showed inhibition of osteoclast formation and resorption and blocking of PTH-dependent calcium mobilization in vivo [110].

Cancer therapeutics

There are different ways to use RGD peptides as cancer therapeutics, depending on the part of the body where the tumor is expressed and its metastatic activity. Some re-

searchers want to interrupt the nutrient pathway of the tumors others attack the tumor itself or the development of metastasic tumors. The cyclic RGD peptide EMD 121974 blocked melanoma cell adhesion without affecting cell viability. As seen above, there might be an effect on endothelial cells as an antiangiogenic therapeutic in metastasis prevention [111]. Antitumor therapy was shown by Carron et al. (1998), who tested a peptidomimetic library in vitro and found one peptidomimetic with an high effectiveness on $\alpha_V\beta_3$ and a 100-fold lower activity on $\alpha_{IIb}\beta_5$. In an animal model the growth of rat leydig cell tumors was inhibited up to 80%, and the development of hypercalcamia was completely blocked [112]. A new collagen-based molecule, tumstatin, with an antiangiogenic N-terminus and antitumor C-terminus, and ligand binding activity for $\alpha_V\beta_3$, was described by Maeshima et al. (2000). During the study an additional $\alpha_V\beta_3$ binding site was found. This additional site was necessary for antiangiogenic activity and not for inhibition of the cell tumor. The authors suggest that binding activity of tumstatin to $\alpha_V\beta_3$ is RGD independent and may involve an additional binding site [113].

Conclusion

This review shows a wide range of peptides used in bone biology. Even though several authors report on linear peptides and cyclic peptides, the state of the art is peptidomimetics. Important integrins that are involved in bone biology and RGD sequence binding are $\alpha_V\beta_3$, $\alpha_V\beta_5$ and $\alpha_{IIb}\beta_3$. We learned that the specificity of RGD peptides is conformation dependent and that adding side chains could change the specificity from endothelial cells to bone cells. This point is very important and should be kept in mind for the therapeutic use of RGD peptides. Very low amounts of peptidomimetics could substitute the former peptides. The approaches shown offer good possibilities for a strategy to generate new therapeutic devices, e. g. for tissue engineering, drugs and drug delivery systems.

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