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PERSPECTIVES IN CLINICAL NEPHROLOGY

Pathophysiology of renal tubular obstruction: Therapeutic role of synthetic RGD peptides in acute renal failure

In his famous work On the Natural Faculties, Galen of Pergamum introduced the idea of "attraction" [δλκηζ] as the principal mode of kidney function: the attraction of blood to the kidney, he claimed, results in the production of urine [1]. Though unproven in Galen's ancient times and entirely rejected by the later generations as a mechanistic explanation of renal function, the concept of attraction has not only survived, albeit in a modified form, but has become one of the cornerstone principles of modern physiology and our current understanding of the pathophysiology of processes as diverse as platelet aggregation, metastases, immune recognition and wound healing, to name a few, all of which are governed by adhesion molecules. Here we review the role of adhesion molecules in the pathophysiology of tubular obstruction, focusing on the integrins and their newly recognized function in it.

The importance of renal tubular obstruction in the pathogenesis of acute renal failure (ARF) was brought to center stage by a series of elegant microdissection studies by Oliver, MacDowell and Tracy [2]. Using servo-null pressure monitoring of the proximal tubular pressure in diverse models of ARF, investigators have provided solid evidence for the elevation in hydrostatic intratubular pressure concomitant with the unchanged stop-flow and estimated glomerular capillary pressures, further confirming the tubulo-obstructive component of this syndrome [3–5]. It has been concluded, therefore, that tubular obstruction and elevated proximal tubular pressure equilibrate glomerular filtration pressure, thus leading to the persisting oliguria. Necrotic epithelial cells have been postulated to provide the matrix for casts obstructing the tubular lumen. Recent findings of viable epithelial cells in the urine of patients and experimental animals with ARF, however, cast doubt on the postulated schema and suggest the possibility of epithelial cell detachment as an important contributor to the development of tubular obstruction [6–8].

In the following discussion we develop this theme, providing data on the possible mechanism(s) of tubular obstruction in ischemic ARF, and on the therapeutic strategies in and benefits of inhibiting tubular obstruction, and suggest some future directions of this fledgling field of investigations.

Integrins: Biochemistry, function, and distribution

Adhesion molecules mediating cell-matrix and cell-cell attachment belong to five families: Ig-like molecules (ICAMs, VCAM-1 and PECAM-1), selectins (L-, P- and E-selectin), carbohydrate ligands for selectins (sialyl Lewisx and GlyCAM-1), cadherins (uvomorulin, or E-CAM, L-, K- and N-CAMs) and integrins. Integrins are noncovalently-bound heterodimeric glycoproteins composed of α and β subunits. To date, 16 distinct α subunits and 8 β subunits have been identified on the protein level [9]. Members of this large family of receptors share several common features. Both subunits have a single hydrophobic transmembrane domain, relatively short cytoplasmic tails and massive extracellular domains. The latter domains are compactly folded by virtue of disulfide bonding, associated together, and both contribute to the formation of the ligand-binding domain. All α subunits contain a sevenfold repeat of a homologous segment, the last three or four repeats of which are likely to contribute to divalent cations binding. The β subunits contain a fourfold cysteine-rich repeat responsible for the folding via internal disulfide groups. Some α subunits, such as the α_1 and α_2, contain a 180 amino acid segment, termed the I domain which, probably imparts some specificity to the receptors. Cytoplasmic domains of the β subunits are indispensable to connecting the receptors to the cytoskeleton. Cytoplasmic tails of β subunits directly bind to talin and α-actinin; the latter can bind directly to actin, thus forming a one-protein link between integrins and cytoskeleton [10].

Integrin receptors are regulated at several levels. The long-term regulation is accomplished through their expression on the cell surface, while the rapid regulation of integrin affinities and specificities is modulated by various extracellular and intracellular signals. These processes are comprehensively discussed in several excellent reviews [11–15], which focus on the role of divalent cations, inside-out and outside-in signaling, and the extracellular matrix in producing conformational changes of the receptor and modulating its function from the inactive to the activated state.

Integrins are abundantly expressed in renal tubules. Immunohistochemical localization of different subunits has revealed that proximal tubules express subunits α_6, α_1 and α_3, that distal tubules express subunits α_1, α_2, α_3 and α_6, and that the β_1 subunits are present along the entire tubule [16–18]. Variations in fixation procedures may have accounted for some of the contentious results regarding the distribution of the α_1 and α_3 subunits along the nephron. Clearly, *in situ* hybridization approach is needed to resolve the existing differences and to gain further insight into the integrins expressed along the nephron.

The above findings on the distribution of integrin receptor subunits along the nephron permit us to make conjectural conclusion as to the nature of heterodimers present. It appears that the proximal tubule expresses $\alpha_1\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_1$ (the possibility of $\alpha_6\beta_4$ expression is less likely, because the β_4 subunit was undetectable in the human kidney [19]). The distal tubule is likely to express, in addition to the above receptors, $\alpha_2\beta_1$ heterodimer.

The binding specificities of individual receptors formally encountered in renal tubules are presented below [11–14]. It should be stressed, however, that the specificities currently assigned to

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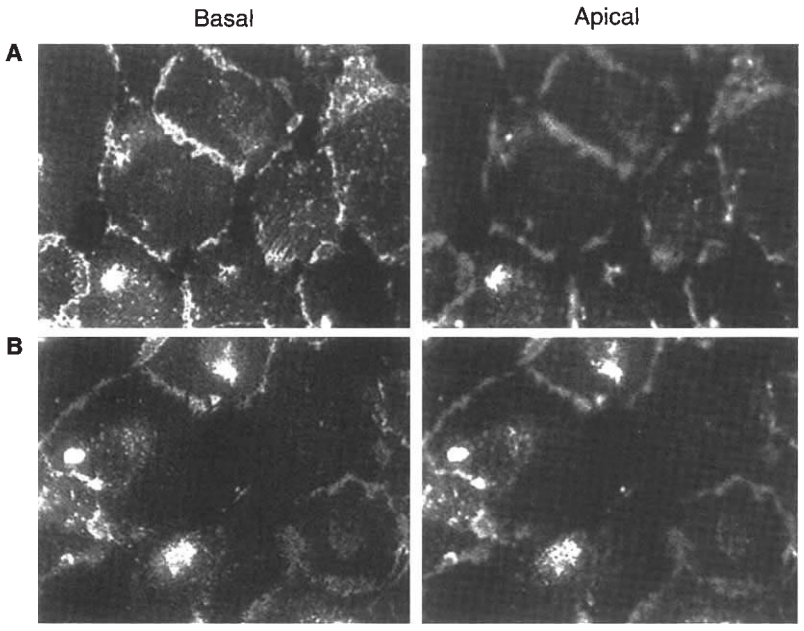

Fig. 1. *Confocal fluorescence microscopy images of the distribution of the α_3 subunit of integrins in BSC-1 cells:* **A** *- intact,* **B** *- exposed to oxidant stress.*

these receptors for the particular matrix proteins may be incomplete. There is compelling evidence that structurally and functionally different isoforms of laminin, collagen and fibronectin exist and that these isoforms are expressed in an organ- and site-specific manner, including various segments of the nephron [20, 21].

$\alpha_1\beta_1$ and $\alpha_2\beta_1$

These heterodimers are receptors for collagen (types I and IV) and for laminin. The $\alpha_2\beta_1$ receptor can also bind to tenascin and, perhaps, to fibronectin. The binding sites for these receptors on collagen type IV are as yet unknown. The $\alpha_2\beta_1$ receptor binds to the collagen type I (possibly the Asp-Gly-Glu-Ala sequence), whereas the respective binding site for the $\alpha_1\beta_1$ receptor remains unknown. It is not clear whether these receptors recognize RGD (Arg-Gly-Asp) ligands or other related sequences containing aspartic acid residue, such as LDV (Leu-Asp-Val) or DGEA (Asp-Gly-Glu-Ala), on collagen or other adhesion substrates.

$\alpha_3\beta_1$

This is a multifunctional receptor for laminin, collagens type I and IV, fibronectin, and epiligrin. The receptor recognizes the RGD (Arg-Gly-Asp) sequence on fibronectin, but probably not on other matrix proteins [22]. When the major fibronectin receptor, $\alpha_5\beta_1$, is present the $\alpha_3\beta_1$ binds poorly to fibronectin, whereas cells readily attach to fibronectin via this receptor in the absence of $\alpha_5\beta_1$. This adhesion is highly enhanced in the presence of Mg^{2+} and Mn^{2+}. The receptor localizes, depending on the conditions of cell culture, either at focal contacts, where it serves as a cell anchor to substratum, or at cell-cell contacts, where it promotes cell aggregation [23]. Additional data on cell-matrix and cell-cell adhesion via this multifunctional receptor are presented below.

$\alpha_6\beta_1$

This is a receptor (activation-dependent in hemapoietic cells) for the E8 fragment of laminin, existing in two isoforms, α_{6A} and α_{6B}, that differ in their cytoplasmic domains. Both receptors interact with laminin isoforms, kalinin and merosin [24].

$\alpha_v\beta_3$

This RGD-recognizing vitronectin receptor has affinity to several other ligands including thrombospondin, von Willebrand factor, fibronectin, and fibrinogen, all of which are important in endothelial cell adhesion, spreading, and migration. Preliminary observations [25] indicate that this integrin is weakly expressed in tubular epithelial cells of human kidneys, and the expression is enhanced in renal tumors. The functional role of the $\alpha_v\beta_3$ integrin receptor in epithelial cells remains unknown.

Redistribution of integrins in stressed epithelial cells

Our previous study of BSC-1 cells subjected to oxidant stress [26] yielded an intriguing observation on the loss of the normal highly polarized distribution of several integrin receptor subunits. Using confocal microscopy, we observed that cells growing as a monolayer exhibited integrins predominantly at the basolateral membranes. This normal pattern underwent a rapid transformation (within 10 to 20 min) from a highly polarized basolateral expression to an ostensibly random distribution between the apical and basolateral membranes upon application of oxidant stress (Fig. 1). Similar observations were made by Lieberthal et al (personal communication) in renal tubular epithelial cells exposed to chemical anoxia. Based on the above findings, we propose a more generalized picture of integrin distribution in stressed epithelial cells (Fig. 2). This view is further buttressed by a series of observations obtained in our laboratory and by other investigators (see below). The cornerstone of the proposed hypothesis is that diverse stress situations result in a loss of the normal polar distribution of integrins. To fully appreciate the mechanisms and the consequences of such a phenomenon, this problem must be considered in detail.

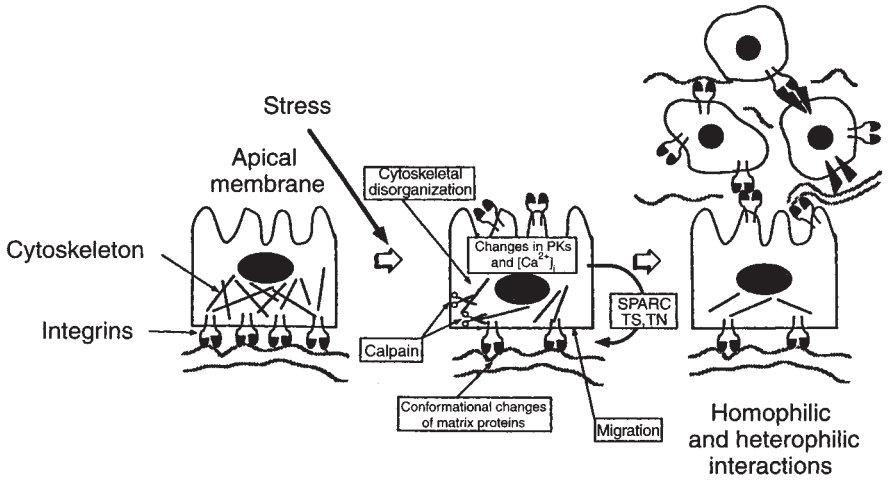

Fig. 2. *Working hypothesis of causes and consequences of the redistribution of integrin receptors from their predominantly basolateral location to the apical cell membrane.* **Left panel.** An epithelial cell with the polarized expression of integrins and their functional coupling to the matrix and to the cytoskeleton. Exposure to oxidant stress or to chemical anoxia result in the redistribution of integrins, as shown in the central panel. Potential causative factors for the loss of integrin polarity are shown in boxes. **Right panel.** Cartoon of the consequences of this event: cell dislodgement and cell-cell interactions. Here, for simplicity of depicting a heterophilic type of interaction, a different class of integrins is shown with triangles. Abbreviations are: PKs, protein kinases; TS, thrombospondin; TN, tenascin; SPARC, secreted protein acidic and rich in cysteine.

Possible mechanisms of the loss of polarized distribution of integrins

Epithelial cell polarity is one of the major attributes of this cellular barrier between the external and internal milieu. The best studied precedent in epithelial cells is related to the stress-induced loss of the polar distribution of Na^+/K^+-ATPase [27]. Other membrane proteins can undergo repolarization or depolarization upon imposition of stress. Integrins in keratinocytes or corneal epithelial cells undergo a series of transformations during the processes of both maturation and wound healing, respectively [28, 29]. The plasticity of the integrins' repertoire and distribution is also exemplified by the malignant transformation of epithelia and the associated changes in the expression of integrins [30, 31]. One of the most striking transformations has been observed in the basal cells of prostate. Normally these cells express α_6, β_1, and β_4 integrin subunits, with the β_1 integrin receptors localized to the basolateral surface of basal cells. In prostate carcinoma, the β_4 integrin subunits are completely lost, while the $\alpha_6\beta_1$ integrins are found in a diffuse pattern on the plasma membrane of basal cells [32]. Hence, the phenomenon of redistribution of the normally polarized integrins occurs under many different circumstances and serve various physiological functions. The mechanisms governing this stress response in renal tubular epithelial cells are, probably, diverse.

Cytoskeletal disruption

Microfilament disruption in proximal tubular cells represents an early event in the pathophysiology of ischemic and nephrotoxic ARF [33, 34]. In their functional mode, integrins constitute a membrane-spanning component in a chain of proteins which binds to particular sites of matrix constituents recognized by individual receptors, and via a group of intermediaries (that is, vinculin, talin and α-actinin) is tethered to the filamentous actin [35]. The entire cascade can be disrupted by interference at any point of connection. By microinjecting the 27-kD proteolytic fragments of α-actinin (these fragments contain the binding site for actin) or the 53-kD fragments (these fragments contain the binding site for the β_1 subunit of integrins) into fibroblastic cells REF 52, BSC-1 and MDCK cells, Pavalko and Burridge [36] observed disassembly of stress fibers at 30 to 60 minutes postinjection, while the focal adhesions remained preserved. By two hours post-injection, only a few focal adhesions were detectable. Similarly, disruption of F-actin with cytochalasin leads to the collapse of focal adhesions [37]. In this vein, stress-induced depolymerization of F-actin [33, 38, 39] may well represent an important mechanism for rapid changes in the binding of integrins to their ligands in the matrix.

State of phosphorylation

It is quite possible that the degree and the site of phosphorylation may regulate the affinity of talin to integrins and integrins to their ligands, thus eventually modulating cell-matrix attachment. Turner, Pavalko and Burridge [40] have demonstrated that the phorbol ester-induced activation of protein kinase C results in a rapid (within 20 min) loss of stress fibers and focal adhesions from BSC-1 cells. These phenomena were accompanied by an increased level of talin, but not vinculin, phosphorylation. Treatment of human fibroblasts with interleukin-1β was associated with rapid phosphorylation and redistribution of talin, both peaking at 15 to 30 minutes, and accompanied by disorganization of the cytoskeleton [41]. In addition to this observation, there is evidence that α-actinin and vinculin, both PIP_2-binding proteins, are substrates for the tyrosine-kinase-activated PLC resulting in hydrolysis of PIP_2 with the subsequent reorganization of the cytoskeleton [42]. Phosphorylation of integrin receptors may also modulate their function. The establishment of focal adhesions via integrin-extracellular ligand binding initiates a cascade of signaling events (Fig. 3). It has been demonstrated that the formation of cell-matrix and cell-cell contacts results in a rapid activation of the Na^+/H^+ exchanger and cell alkalinization [43, 44], elevation of cytosolic calcium concentration, possibly, due to the activation of a 50-kDa β_3 integrin-associated protein, presumed to represent an integrin-regulated calcium channel in endothelial cells and neutrophils [45, 46], delayed stimulation of a K^+ current [47], and a series of tyrosine phosphorylation reactions mediated via activation of focal adhesion kinase [48, 49]. Conversely, the reduction of cell-substrate adhesion and detachment of cells from their matrix is associated with the activation of a phosphotyrosine phosphatase and the decreased tyrosine phosphorylation of focal adhesions [50]. It has been shown that the phosphorylated form of the β_1

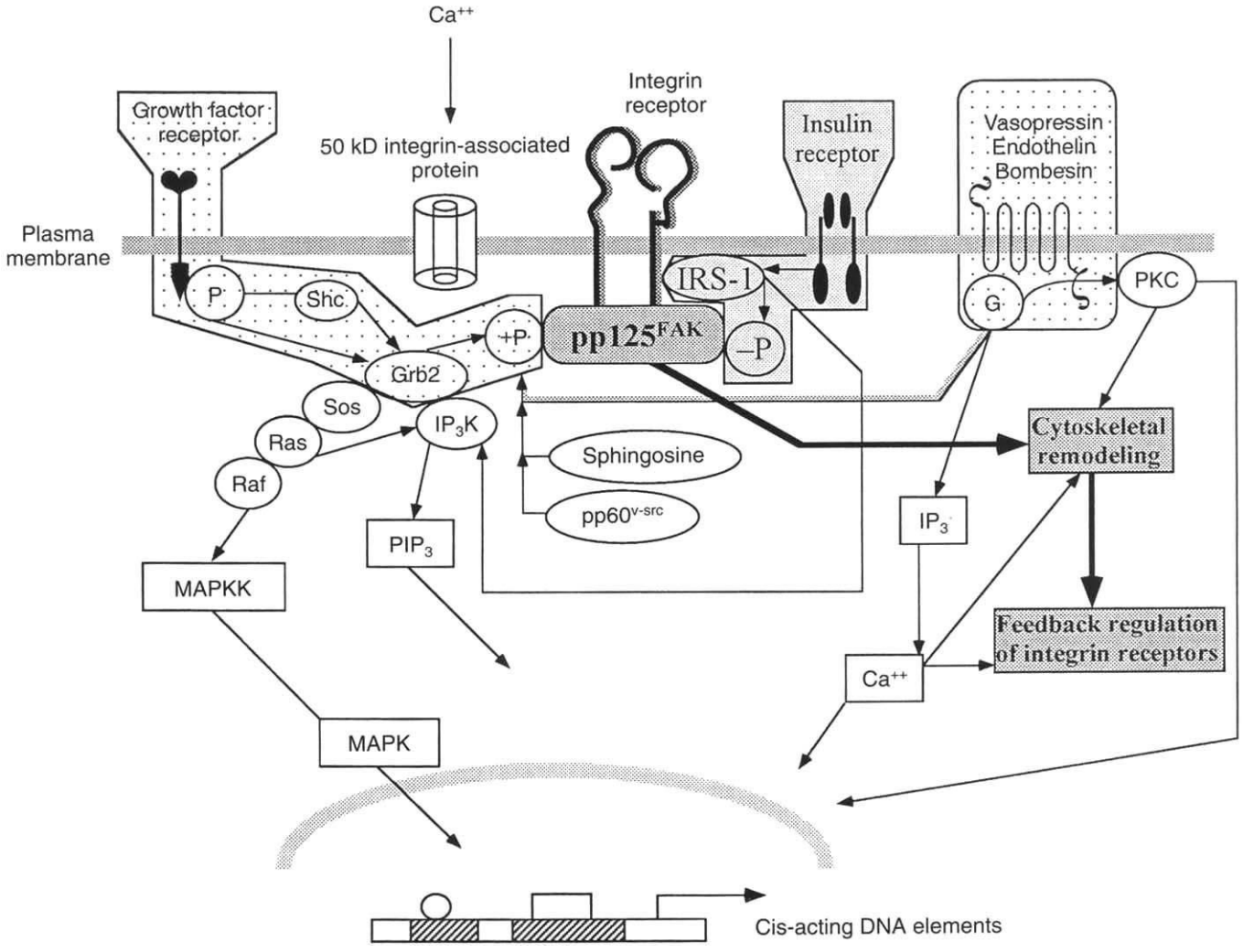

Fig. 3. *Integrin signaling cascades and interactions with other signaling systems.* Interaction of integrin receptors with focal adhesion kinase leading to cell remodeling and, possibly, to feedback regulation of integrins is depicted with thick arrows. Potential interactions between integrin receptors and various growth factors (such as epidermal growth factor, EGF, or insulin and insulin receptor substrate-1, IRS-1) are outlined with dots. Actions of serpentine G-protein-coupled receptors on the elements of integrin signaling are shown on the right (dotted area). See the text for details.

integrin subunit has different subcellular localization than the nonphosphorylated form, suggesting that tyrosine phosphorylation is involved in cellular traffic of the receptor [51]. The observed versatility of signaling mechanisms triggered by the establishment or dissolution of focal adhesions can provide the means for the conformational changes of proteins composing the focal adhesions (such as the β_1 integrin, paxillin, and tensin, all containing phosphotyrosine) and explain the phenomena of affinity modulation of integrins toward the extracellular matrix proteins, outside the cell, and toward the cytoskeletal elements, inside the cell [52].

The list of the substrates for tyrosine kinase phosphorylation is continuously growing, and presently includes vinculin, talin, tensin, paxillin, integrins, pp125^{FAK}, cadherins, and catenins. pp125^{FAK} represents so far the best studied tyrosine kinase associated with focal adhesions. This protein is autophosphorylated during cell adhesion (the cytoplasmic domain of β subunits is necessary for this reaction), but it also undergoes autophosphorylation in response to pp60$^{v\text{-}src}$, bradykinin, endothelin, and sphingosine, resulting in the increase in its tyrosine kinase activity. Dephosphorylation of pp125^{FAK} may be accomplished in part via activation of the SH2 domain-containing protein tyrosine phosphatase Syp (SHPTP2) which is activated by the insulin receptor substrate-1 (IRS-1). This IRS-1 *per se* can be associated with integrin receptor $\alpha_v\beta_3$ [53], thus providing the long-sought link between growth factors and integrins. In Rat-1 fibroblasts expressing insulin receptor, insulin treatment resulted in dephosphorylation of pp125^{FAK}, whereas in fibroblasts transfected with insulin receptor cDNAs lacking the C-terminal twin tyrosine phosphorylation sites the degree of pp125^{FAK} phosphorylation was unaffected by insulin [54]. In turn, Grb2 and PI [3]-kinase are two known substrates of pp125^{FAK} [55, 56]. Collectively, these studies interconnect integrins with receptors for hormones and growth factors, and implicate the state of pp125^{FAK} phosphorylation in modulation of the Ras/MAPK and PIP_3 signal transduction pathways.

Activation of proteases

Components of integrin receptors, together with talin and α-actinin, are known substrates for proteases. It has been demonstrated that at least one calcium-activated neutral protease, calpain, co-localizes with the elements of focal adhesions [57]. Cleavage of integrin receptor subunits by calpain, such as the cytoplasmic domain of the β_4 subunit, has previously been documented [58]. In our experiments, pretreatment of BSC-1 cells with an inhibitor of calpain, MDL 28170, resulted in a dramatic decrease in the number of BSC-1 cells detached from the matrix upon exposure to oxidant stress (unpublished observations). The potential role of calpain and other proteases deserves further investigation.

Induction of NO synthase

Increased NO release has been implicated in the pathophysiology of ARF [59]. NO synthases, both constitutive and inducible forms, are expressed in tubular epithelial cells and in several cell lines derived from renal tubular epithelia [60, 61]. As a powerful oxidant, NO can affect adhesion of epithelial cells to their matrices [62]. Our laboratory has observed that, in BSC-1 cells exposed to oxidant stress, pretreatment with antisense oligodeoxynucleotides directed to the initiation codon of the inducible NO synthase partially prevented cell detachment and attenuated cell death (Peresleni et al, unpublished observations).

Modification or conformation of matrix proteins

Changes in availability and composition of ECM are the known modulator of integrins' affinity [63]. Different stress situations have been reported to alter matrix proteins. Hydroxyl radicals damage fibronectin by causing the loss of tryptophane, the increase in bityrosine and extensive crosslinking [64]. These changes carry a potential to affect cell-matrix adhesion.

Cell locomotion

Since the classical studies by Abercrombie, Heaysman and Pegrum [65], the idea of a redistribution and directional movement of membrane proteins during cell locomotion has gained solid experimental support. Several membrane-spanning proteins expressed on the basal surface of the cell are retrieved and re-expressed on the apical surface during cell migration. Since the integrity of epithelial cell monolayers subjected to stress becomes impaired and intercellular gaps are formed, the cells migrate inwardly to the voids and seal them [66]. Using confocal microscopy of BSC-1 cells labeled with a biotinylated RGD peptide, we observed that the binding sites, ordinarily expressed on the basal membrane, were expressed on the apical membrane of the cells migrating into a wound (MSG, unpublished observations). Therefore, it is conceivable that the loss of polarity in the expression of integrins may be due, at least in part, to the process of epithelial cell migration, and that the locomoting cells, compared to the rest of the population, are less tightly attached to the matrix and express integrins on the apical surface more abundantly.

Endogenous antiadhesins and repulsins

There is accruing evidence that many cells synthesize and secrete substances which, in a paracrine or autocrine fashion, interfere with cell-matrix adhesion. One of the best studied secretory products is the extracellular matrix-associated glycoprotein SPARC (acronym for "secreted protein acidic and rich in cystein," also termed osteonectin, BM-40 or 43-kD protein) which selectively disrupts focal adhesions [67]. This antiadhesin and its mRNA are expressed in the fetal and adult kidney, and are induced in stress situations and in tissues undergoing remodeling and repair. Together with other known antiadhesins, thrombospondin and tenascin, SPARC may be a candidate molecule contributing to the detachment of stressed renal tubular epithelia. This speculation clearly warrants future testing. Cytotactin is an example of a growing family of repulsins. This molecule functions amphitropically, as it can mediate cell adhesion, but can also discourage cells from attachment and spreading [reviewed in 68]. The role of these molecules as candidates for modulation of renal tubular epithelial cells adhesion calls for investigation.

The functional consequences of the loss of polarized distribution of integrins in renal tubular epithelial cells may be twofold. First, cell detachment from the matrix is a well-established consequence of the dysfunction of several integrins. Carter et al [69] have demonstrated that anti-$\alpha_3\beta_1$ monoclonal antibody P1B5 detached human keratinocytes, which express this receptor, from their laminin- or fibronectin-containing substratum. Similar detachment is observed in BSC-1 cells exposed to this antibody or to the cyclic RGD peptide, 10 to 50 μM (MSG, personal observation). There is emerging evidence that disruption of epithelial cell-matrix interactions leading to the detachment of these anchorage-dependent cells, or anoikis (Greek for homelessness), induces programmed cell death [70–72]. Hence, renal tubular cell detachment from the matrix is a likely consequence of integrin redistribution from the basal cell surface. Second, the redistribution of integrins from the basal to the apical cell membrane predisposes *in situ* cells to promiscuous interactions with the dislodged cells or with matrix fragments, as the evidence presented below demonstrates.

Model of tubular obstruction: Cell-cell and cell-matrix-cell adhesion

The fate of the dislodged tubular epithelial cells must now be addressed. As Racusen et al [6] and Graber et al [7] have pointed out, viable cells can be harvested from the urine of patients with ARF. On the other hand, based on microdissection studies by Oliver et al [2], it is known that a proportion of tubules shows partial or complete obstruction. It is, therefore, reasonable to assume that some of these dislodged tubular epithelial cells become aggregated to form tubular casts. The real question is, How does this occur?

We have considered the possibility that integrins expressed on the apical surface of tubular epithelia can promote cell-cell adhesion when presented with a dislodged cell. The major prerequisite for such a mechanism to take place is the functional competence of the apically expressed integrins. Indeed, using cell-cell adhesion assays, together with the measurements of matrix-coated latex beads' attachment to the stressed BSC-1 cells, we have demonstrated a significant increase in cell-cell and cell-bead attachment to monolayers which lost their polar distribution of integrins [73]. Hence, the ability of apical integrins to mediate adhesion should not be the limiting factor in cellular aggregation.

The possible cellular interactions – homophilic and heterophilic, as well as via fragments of matrix proteins – are cartooned

in Figure 2. The very fact that this process is inhibited, at least in part, by RGD peptides ([73] and see below) narrows the number of participating molecular candidates. In accord with our previous observation on the expression of the $\alpha_3\beta_1$ multifunctional receptor on the apical surface of stressed BSC-1 cells [26], the possibility of homophilic interactions via this receptor should be considered. In human keratinocytes, cell-cell adhesion was inhibited by the P1B5 antibody against the α_3 subunit or with the monoclonal anti-β_1 antibody P4C10 [69]. The relocation of the integrins from focal contacts to cell-cell contacts was documented, a fact supportive of either type of interaction, homo- or heterophilic. By transfecting human α_3 cDNA into K562 cells, normally lacking the endogenous α_3, Weitzman et al [74] were able to demonstrate that the transfectants accomplished adhesion, spreading, and homophilic cell-cell aggregation via the $\alpha_3\beta_1$ receptors. Homophilic interactions via the $\alpha_3\beta_1$ receptor were further established in studies of MG-63 human osteosarcoma cells which express this receptor. The immobilized receptor supported the adhesion of these cells, the process which was inhibited by monoclonal antibodies against both α_3 and β_1 subunits [75]. In addition, only $\alpha_3\beta_1$ integrin from detergent extracts of the cells specifically binds to the affinity matrix containing the purified $\alpha_3\beta_1$ receptors [75]. Larjava et al [76] have documented that the $\alpha_2\beta_1$, $\alpha_3\beta_1$, and $\alpha_5\beta_1$ integrins were clustered in areas of cell-cell contacts. These investigators suggested that in epithelial cells the above receptors, in addition to their known role in substrate adhesion, can also function in cell-cell interaction.

Another possible scenario for the RGD-sensitive cell-cell interaction is suggested by the recent studies employing the β_1-activating antibody TS2/16 [77]. B lymphocyte cell lines expressing the $\alpha_4\beta_1$ integrin, which has no apparent affinity to RGD peptides, acquired the capacity to recognize the RGD sequence after stimulation with TS2/16 antibody. This activating antibody also enhanced attachment of cells transfected with human α_3 cDNA, KA3 cells, to fibronectin [74]. Unfortunately, the effect of RGD peptides on the attachment of these transfectants has not yet been examined.

The possibility of interaction between the opposing receptors via fragments of matrix proteins was considered in the study by Larjava et al [76]: neither fibronectin, laminin, nor type IV collagen co-localized to cell-cell contacts. Hence, the existing precedent in other epithelial cells supports the involvement of homophilic and, less likely, heterophilic interactions in renal tubular cell aggregation. Although a contribution of cadherins to this cell-cell adhesion is possible, especially in view of the existing sequence homology between cadherins and integrins [78], this type of adhesion would be resistant to RGD peptides and, therefore, is beyond the scope of the present review.

As mentioned above, cell-cell interaction via integrin receptors can be mediated, at least in part, by the soluble fragments of matrix present in the urine or, alternatively, through matrix fragments torn from the basement membrane during the process of cell detachment. Together with the known components of the tubular basement membrane, other RGD-containing proteins, such as osteopontin, Tamm-Horsfall protein, or IGF-binding protein, may participate in the bridging integrin receptors of neighboring cells. Finally, the possibility that the dislodged cells may attach to the denuded basement membrane, in a way similar to the adhesion of platelets to the sites of endothelial cell damage and loss, has not been excluded. These possibilities are addressed below in our discussion of *in vivo* experiments with two cyclic RGD peptides characterized by vastly different potency in inhibiting cell-matrix adhesion.

The above *in vitro* data and conclusions have been further tested by monitoring the proximal tubular pressure in rats with an ischemic model of ARF. One of us (GFD) has consistently observed the elevation of proximal tubular pressure in rats after the release of renal artery occlusion. When a linear RGD peptide was infused into the renal artery, however, the elevation of proximal tubular pressure was curtailed, the effect of which was not reproduced by the inactive peptide or by a vehicle alone [73]. Our recent immunohistochemical studies of ischemic kidneys (Romanov et al, manuscript in preparation) have shown the appearance of the immunodetectable β_1 subunit, usually localized to the basal aspect of tubular epithelia, on the luminal surface of tubular epithelial cells and on the surface of desquamated and conglomerated cells within tubular lumen. These observations are consistent with the proposed role of integrins in triggering the cascade of events leading to cellular detachment and aggregation of detached cells. It is important to bear in mind that all these interactions leading to cellular agglomeration were inhibited by synthetic RGD peptides.

Disintegrins and synthetic cyclic RGD peptides

Disintegrins comprise a large family of proteins containing the RGD motif or a related sequence with a high affinity to integrin receptors. Initially, these proteins were purified from snake venoms. Scarborough et al have screened sixty-two snake venoms in search of a potent inhibitor of platelet aggregation [79]. Fifty-two of them inhibited glycoprotein (GP) IIb-IIIa-mediated platelet adhesion, but only one of them, termed barbourin (from the southeastern US pigmy rattlesnake *Sistrurus m. barbouri*), was specific for the GPIIb-IIIa versus other integrins. Surprisingly, this peptide, despite its high structural similarity to other viper venoms with disintegrin properties, did not contain the RGD sequence and instead possessed the sequence Lys-Gly-Asp (KGD).

Chemical synthesis of RGD peptides intends to mimic the properties of disintegrins. Linear RGD-containing peptides do not guarantee the necessary structural stability and, thus, are usually less potent than their venom analogs. In addition, it is known that linear RGD peptides (such as GRGDS and GRGDNP) are rapidly excreted and degraded *in vivo* [80]. Therefore, several strategies such as polymerization or complexation with macromolecules have been employed in the past to improve the biological properties of such peptides. However, these strategies invariably led to the production of large molecules which *a priori* would not pass the glomerular filtration barrier to reach the tubular lumen. An alternative strategy of cyclization of RGD peptides has been shown to provide the necessary structural constraints and, thus, resulted in a significant increase in their affinity for integrins and inhibitory potency [80–82] without compromising the filterability of the peptides.

We have previously characterized two cyclic RGD peptides with contrasting properties in BSC-1 cell-matrix and cell-cell adhesion [83]. RGDDFV exerted a potent inhibitory effect on BSC-1 cell-matrix adhesion: the IC_{50} for adhesion to fibronectin and laminin was 2 μM and 250 μM, respectively. RGDDFLG was less potent in inhibiting BSC-1 cell adhesion to fibronectin and laminin: the maximal concentration of peptide used in these assays resulted in a 58% inhibition of adhesion to fibronectin and a 69% inhibition of adhesion to laminin. RDADFV, a cyclic peptide which does not contain the RGD sequence, thus useful as

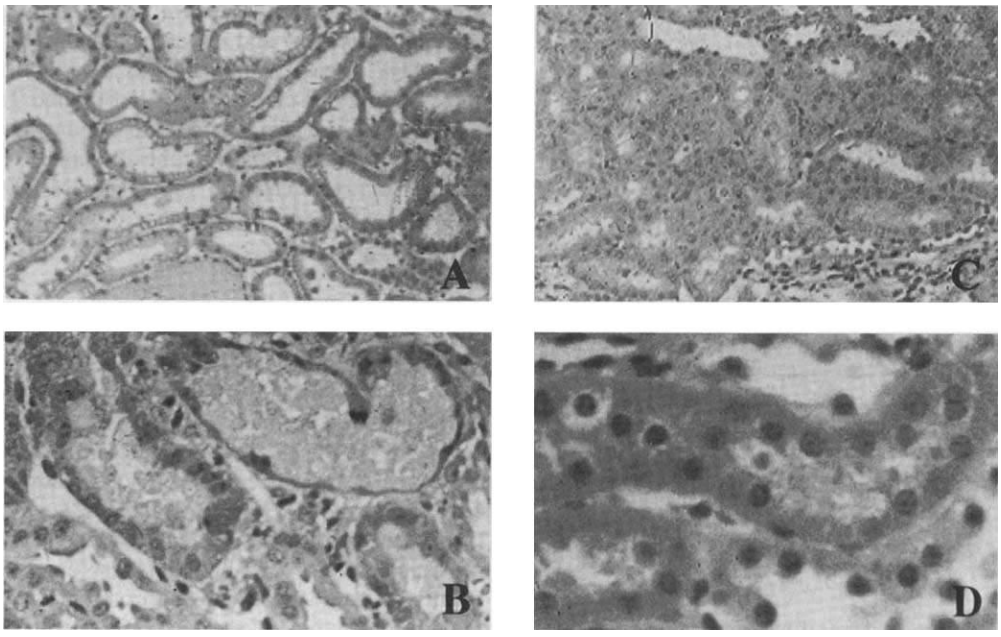

Fig. 4. *Histologic findings in postischemic kidneys 3 days after ischemia.* **A** and **B**. Untreated ischemic kidneys. **C** and **D**. RGDDFV-treated ischemic kidneys. Cyclic RGD peptide-treated kidneys show virtually no tubular dilatation and a very mild tubular obstruction, compared with the severely dilatated and obstructed tubules in the nontreated group. Occasional necrotic and dividing tubular epithelial cells *in situ* are observed with equal frequency in both groups. Magnification ×100 (A and C) and ×400 (B and D).

a control, did not affect cell-matrix adhesion. Using PepTite 2000 as adhesion substrate, RGDDFLG (25 μM) did not affect BSC-1 cell adhesion, whereas an equimolar concentration of RGDDFV inhibited 90% adhesion [83].

BSC-1 cells exhibited a strong tendency for self-conglomeration in suspension: the number of single cells progressively decreased with time in suspension, while the number of conglomerated cells progressively increased [83]. When cell-cell adhesion assays were performed in the presence of the cyclic RGD peptides at a concentration similar to that used *in vivo*, the process of cellular aggregation was virtually abolished. Collectively, these findings demonstrated that cyclic RGD peptides used in these studies affected cell-matrix and cell-cell adhesion with different potencies; although differing in effectiveness in inhibiting cell-matrix adhesion, both peptides were equipotent in the cell-cell adhesion assay. Based on profound differences in the inhibitory potency of these peptides, we utilized them for in vivo experiments (see below) at the concentration (~4 μM) which proved to be effective for RGDDFV, but ineffective for RGDDFLG.

Effects of cyclic peptides on the course of ischemic acute renal failure

The systemic effects of these cyclic peptides were examined *in vivo* in a rat model of ischemic ARF [83]. Cyclic RGDDFLG- and RGDDFV-treated groups showed a significantly lesser retention of Cr, as well as an accelerated recovery of C_{Cr} ($P < 0.05$) on postoperative day 1, compared to both control groups (animals treated with either the inactive cyclic peptide or with the vehicle). In addition, a significant difference in C_{Cr} between cyclic RGDDFLG- or RGDDFV-treated and cyclic RDADFV-treated groups was observed on postoperative day 3. These data indicate that the recovery of renal function after ischemic injury occurred faster in cyclic RGDDFV-treated (2 days) or RGDDFLG-treated (3 days) groups, compared to animals treated with either the inactive peptide or with the vehicle ($P < 0.05$). The rank order for the cyclic peptides in ameliorating ischemic ARF and accelerating recovery of C_{Cr} was as follows: cyclic RGDDFV ≥ RGDDFLG ≫ RDADFV.

Microscopic examination of the urinary sediments revealed striking differences between the experimental and control groups. While control urine samples showed conglomerates of epithelial cells on postischemic day 1, the urinary sediments of RGDDFLG- or RGDDFV-treated animals displayed large numbers of dispersed cells.

Histologic examination of rat kidneys obtained from animals treated with the RGDDFV peptide or with the vehicle revealed striking differences in the degree of tubular obstruction. Three days after acute ischemia, treated kidneys showed no tubular dilatation and a very mild tubular obstruction, compared with the severely dilatated and obstructed tubules in the nontreated group. Occasional necrotic and dividing tubular epithelial cells *in situ* were observed with equal frequency in both groups (Fig. 4). These data suggest that cyclic RGD peptides do not significantly affect the fate of tubular cells in terms of necrosis or mitogenesis, but acted rather by preventing tubular obstruction. This conclusion is consistent with the other findings.

The time course of the effect of cyclic peptides was examined in rats subjected to 45 minutes renal artery occlusion and injected with the peptide at 2 and eight hours after the reperfusion commenced. As shown in Figure 5, when RGD peptides were injected at two hours postischemia, the recovery of creatinine clearance was accelerated to the same extent as it was with the peptide injection immediately after the release of a clamp. This effect, although it persisted in a group of animals that received the peptide after eight hours, was less pronounced, compared with the two hour group. It is possible that the adhesive events critical for the subsequent tubular obstruction occur early after an ischemic

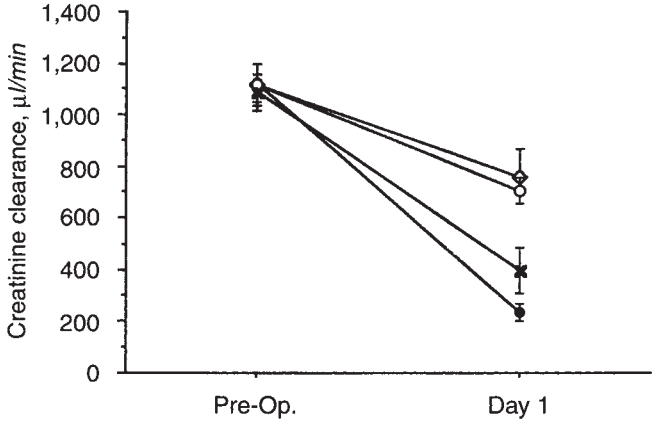

Fig. 5. *Dependence of creatinine clearance on the timing of injection of cyclic RGD peptide.* Measurements were performed 24 hours post-ischemia. Symbols are: (◇) immediate injection; (○), (×) injection was made 2 and 8 hours, respectively, after release of renal artery clamp; (●) control ischemia. $P < 0.05$ was observed between (◇, ○, and ×) vs., (●); between (○) and (×), (●).

episode, when they can be prevented by administration of cyclic RGD peptides.

The observed effects of cyclic RGD peptides were possibly mediated by their tubular effects. Given that the molecular weights of these cyclic peptides are less than 900 Da, and their electrical charge is close to neutral, it is reasonable to assume that the peptides should readily undergo glomerular filtration [84, 85]. To prove that a systemically injected RGD peptide indeed appears in the tubular lumen, where it could act on the desquamated cells, we next examined urine sediments obtained from rats subjected to renal ischemia and administered the biotinylated RGD peptide, RGD[Bt-K^5]. Urinary samples collected at three hours postoperatively and stained with Rhodamine B streptavidin revealed the presence of the biotinylated ligand on the surface of fixed non-permeabilized cells in the urine sediment. These data are consistent with the appearance of RGD peptides in the tubular lumen, probably as a result of their glomerular filtration. The data obtained with radiolabeled peptides confirmed and further expanded these observations (see below).

These studies demonstrated that a single systemic administration of cyclic peptides *in vivo* ameliorated ischemic ARF, probably, through their inhibitory action on cell-cell conglomeration in the tubular lumen. *In vivo* studies were performed using these peptides at a calculated systemic concentration approximately ten times lower than the IC_{50} established by the *in vitro* laminin adhesion assay. Even at this apparently lower concentration (it is not excluded, however, that the peptides are concentrated in the tubular lumen), both cyclic RGD peptides significantly reduced retention of Cr and accelerated C_{Cr} recovery in the postischemic period. Hence, our observations establish that cyclic peptides containing the RGD sequence ameliorate ischemic ARF. It is reasonable to suggest that cyclic RGDDFLG and RGDDFV peptides were effective in inhibiting tubular obstruction by predominantly preventing cell-cell adhesion, rather than cell-matrix adhesion. Such an inference is based on four independent lines of experimental data. First, the peptides were used at concentrations which were only partially inhibitory for cell-matrix adhesion in the case of cyclic RGDDFV and non-inhibitory in the case of cyclic RGDDFLG. Second, comparison of two cyclic RGD peptides with vastly different inhibitory potencies in the cell-matrix adhesion assay showed that they were equipotent in preventing cell-cell aggregation *in vitro* and almost equipotent in ameliorating ischemic ARF *in vivo*. Third, analysis of urine sediments from the cyclic RGDDFV- and RGDDFLG-treated groups showed dispersed cells, in striking contrast to the massive cellular conglomerates seen in the urine of control animals. Finally, histological data in rats treated with the cyclic RGDDFV demonstrated abolition of tubular obstruction without any detectable effect on the frequency of necrotic or mitotic cells or the degree of denudation.

Recently, several strategies to manipulate adhesion molecules during the course of ischemic injury have been proposed. One approach explores the therapeutic effects of anti-ICAM-1 and anti-LFA-1 antibodies. It has been demonstrated that these antibodies are effective in an acute ischemic myocardial reperfusion model [86, 87]. More recent observations have provided data on the therapeutic utility of these antibodies in protection against renal ischemia [88]. Most probably, these effects are confined to the inhibition of leukocyte migration and/or blood coagulation in the renal microvascular bed, thus resulting in improved hemodynamics. In our experiments, thrombotic complications of the prolonged clamping of the renal artery were negated by virtue of anticoagulation with heparin performed prior to 45′ ischemia. The approach described in the present work utilizing small, ultrafilterable and less immunogenic peptides is directed toward inhibition of tubular obstruction, thus aiming to improve urodynamics. Future attempts to combine both approaches may lead to the potentiaton of their effectiveness.

We have recently explored the effect of cyclic RGD peptides in another model of ARF, specifically, warm ischemia-induced ARF after orthotopic kidney autotransplantation in pigs [89]. In an attempt to emulate real harvesting conditions, bilaterally nephrectomized kidneys were exposed to 20 minutes warm ischemia and stored on ice for 24 hours. Anephric pigs developed severe uremia by the time of autotransplantation. Harvested kidneys were briefly perfused with cyclic RGD peptide RGDDFV at the concentration of 5 μM or with the vehicle, and the renal artery was anastomosed end-to-side with the aorta. Ureters were diverted to the skin to ease the clearance studies performed for three consecutive days after autotransplantation. Virtually all animals were anuric on the day 1 of autotransplantation. Despite the significant variability in the severity of ARF in individual successful cases, on postoperative days 2 and 3, C_{Cr} was consistently higher on the side of peptide infusion compared to the internal control infused with the vehicle only.

Half-life of RGD peptides in the circulation and their metabolic clearance

Our next goal was to synthesize RGD peptides that can be successfully tagged with radioactive indicators. Synthetic Gly-Arg-Gly-Asp-Ser-Pro-Cys (GRGDSPC) peptide containing the C-terminal cystein was used for the conjugation with ^{99m}Tc pertechnetate (manuscript submitted).

^{99m}Tc-GRGDSPC (30 μCi/0.3 ml) was injected in a tail vein of normal Sprague-Dawley rats, the animals were sacrificed at different times postinjection, and the radioactivity accumulated in different organs was detected with a well-type gamma counter and expressed on per organ or per gram basis. These procedures were repeated in rats subjected to the 45 minutes renal ischemia followed by the contralateral nephrectomy. The clearance of

blood in both groups of animals was rapid and indistinguishable between the control and ARF rats (at 10 min postinjection, the radioactivity of the blood accounted for 12.91 ± 1.47% and 11.17 ± 2.13% of the injected dose, respectively). In controls, kidneys accumulated the major portion of the injected radioactivity by 10 minutes postinjection 12.47 ± 0.47% (both kidneys), with the gut and liver retaining 6.58 ± 0.99% and 5.19 ± 0.49% of the injected dose, respectively. In ARF group, renal accumulation accounted for 3.53 ± 1.24% (one kidney), while the gut and liver accumulation accounted for 6.72 ± 0.79% and 3.31 ± 0.44%, respectively. The other organs (brain, lungs, heart, spleen, and stomach) contained less than 1% of the injected dose in both groups of animals. By 180 minutes postinjection, the gut content of ^{99m}Tc amounted to 8.62 ± 0.92% injected dose in control animals, while the ARF rats showed a significant increase in the amount of the isotope accumulated in the gut (19.50 ± 2.16% of injected dose, $P < 0.05$ vs. timed control). This suggests that in ARF rats, the gut becomes a significant route for the elimination of ^{99m}Tc-GRGDSPC. When the data were expressed per g wet tissue weight, the kidneys retained the largest portion of the injected radioactivity at 10 minutes postinjection: 8.42 ± 0.18% of injected dose/g wet wt in control and 4.42 ± 1.88% of injected dose/g wet wt in ARF rats, $P < 0.05$). These data indicate that the renal excretion of the peptide represents the predominant and specific route for its elimination.

Given the vastly different renal blood flow and glomerular filtration rate in control and ARF rats, we next examined the relative accumulation of ^{99m}Tc-GRGDSPC in both groups. The experimental protocol was modified by the double isotope injection of ^{111}In-diethylenetriaminepentaacetic acid (DTPA) five minutes prior to the sacrifice. The results were expressed as the ratio of the activities ^{99m}Tc-GRGDSPC/^{111}In-DTPA, thus normalizing the amount of the accumulated RGD peptide to the glomerular filtration rate [90]. At 60 minutes postinjection of ^{99m}Tc-GRGDSPC, the ratio RGD/DTPA in control kidneys equaled 4.03, while it was more than four times higher in ARF rats: 17.4. These data indicate that postischemic kidneys retain ^{99m}Tc-labeled RGD peptide compared to the normal kidney. Taking into account our previous findings on the desquamation of tubular epithelial cells and exposure of the integrin receptors, normally occupied by the matrix, to the exogenously provided RGD ligands, the above data are consistent with the specific binding of the injected ^{99m}Tc-labeled RGD peptide to these exposed integrins. Based on these results, it is quite plausible that the ratio ^{99m}Tc-RGD:^{111}-In-DTPA may serve as a sensitive diagnostic parameter for detection of tubular obstruction in this and other pathological conditions.

Potential side effects of RGD peptides

As an integral part of various matrix proteins, RGD-containing peptides, at least theoretically, should be well tolerated. To date, no detailed toxicological studies of these peptides have been published. Based on the available physiological data, the following potential side effects should be considered.

Platelet dysfunction may represent one possible complication of systemic administration of RGD peptides. In *in vitro* experiments on binding of the biotinylated $\alpha_{IIb}\beta_3$ integrin to immobilized fibrinogen, linear GRGDS peptide showed IC_{50} equal to 10 μM, whereas the cyclic RGDFV peptide was ten times more potent [81]. *In vitro* platelet aggregation examined in the presence of different snake venoms yielded the following information: barbourin, tergeminin and eristicophin inhibited the ADP-induced aggregation with IC_{50} equal to 309 nM, 192 nM and 104 nM, respectively. Clearly, these venom derivatives are much more potent in inhibiting platelet aggregation than cyclic RGD peptides. Although we have not studied platelet aggregation specifically, there were no clinical manifestations of coagulopathy in our *in vivo* experiments with rats and pigs.

Cell migration may represent another target for RGD peptides. It has been shown that this process was inhibited *in vitro* by linear RGD peptides with half-maximal effect at the concentration of ~10 μM [66]. Given the importance of cell migration in restoration of tubular epithelial integrity and barrier function during postischemic period, when the denuded basement membrane has to be sealed by migrating neighboring cells remaining *in situ*, this potential interference should be seriously considered. In this respect, the concentration of RGD peptides is of critical importance. Our experimental use *in vivo* cyclic RGD peptides at a concentration of 4 to 5 μM, by far lower than that reported to inhibit cell migration in other systems (such as inhibition of BSC-1 cell migration at ~10 μM [66], of human keratinocytes at 100 μM [91]), not only did not inhibit the recovery from ischemic insult, but significantly accelerated it. Such an effect would be inconsistent with the possibility of an RGD peptide interfering with the epithelial wound healing process.

Concluding remarks and future perspectives

This brief review takes stock of the most recent investigations on the role of integrins in the pathophysiology of ARF. The data presented above incriminate the redistribution of integrins in renal tubular epithelial cells in the development of cell desquamation, aggregation and tubular obstruction. Curiously, several passages in Hippocrates' *Aphorisms*, though somewhat obscure today, suggest the amazing prediction of such a mechanism, as in his observation that "[w]hen small fleshy substances like hairs are discharged along with thick urine, these substances come from the kidneys."

We have also summarized compelling evidence, from both in *vitro* and *in vivo* experiments, that RGD peptides moderate the severity of ARF by inhibiting tubular obstruction. This effect was observed when the peptides were injected either prior to the release of the clamp or two hours later, but was blunted when RGD peptides were injected eight hours later. These findings suggest that RGD peptides should be further evaluated as potential therapeutic tools in the management of ARF.

Several targets for future investigations clearly emerge from this review. The cellular mechanisms leading to the loss of polar distribution of integrins in stressed renal tubular epithelial cells, the precise identity of integrins participating in this process, as well as the modes of cell-cell interaction leading to the obstruction of tubular lumen remain to be elucidated. Any potential side effects of RGD peptides should be thoroughly investigated. Clearly, broad toxicological studies of RGD peptides ought to be performed before any attempts to introduce them into the management of selected forms of ARF are undertaken.

Eisei Noiri, Victor Romanov, Thomas Forest, James Gailit, Gerald F. DiBona, Frederick Miller, Prantika Som, Zvi H. Oster and Michael S. Goligorsky

Departments of Medicine, Physiology & Biophysics, Pathology, and Nuclear Medicine, State University of New York at Stony Brook, Stony Brook, New York; Brookhaven National Laboratory, Upton, New York; National Cancer Institute, Frederick, Maryland; and Department of Internal Medicine, Iowa Medical College, Iowa City, Iowa, USA

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Reprint requests to Michael S. Goligorsky, M.D., Ph.D., Division of Nephrology, SUNY at Stony Brook, New York 11794-8152, USA. E-mail: mgoligorsky@epo.som.sunysb.edu

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