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Pathogenetic role of Arg-Gly-Asp-recognizing integrins in acute renal failure

(adhesion molecules/matrix proteins/tubular obstruction/proximal tubular pressure/kidney)

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ABSTRACT **Reorientation of the α_3 subunit of integrins from predominantly basal to the apical cell surface of cultured renal tubular epithelial cells subjected to oxidant stress has previously been demonstrated. The present study was designed to assess functional competence of ectopically expressed apical integrins. Cell–cell adhesion assay revealed enhanced cytoattractant properties of stressed cells. Stressed epithelial cells exhibited specific recognition and binding of laminin-coated latex beads. These processes were inhibited with the peptide Gly-Arg-Gly-Asp-Asn-Pro (GRGDNP) suggesting a role of RGD-recognizing integrins in augmented adhesion to stressed cells. Given that such enhanced adhesion in *in vivo* acute renal failure may govern tubular obstruction by desquamated epithelium, a physiological marker of patency of tubular lumen, proximal tubular pressure, was monitored in rats subjected to 60 min of renal ischemia followed by reperfusion. Proximal tubular pressure increased 2-fold after 2 hr of reperfusion in animals that had undergone 60 min of ischemia. Infusion of GRGDNP into the renal artery during reperfusion period virtually abolished an increase in proximal tubular pressure observed in ischemic acute renal failure. These *in vitro* and *in vivo* findings are consistent with the hypothesis that RGD-recognizing integrins play an important role in the pathogenesis of tubular obstruction in ischemic acute renal failure.**

Acute renal failure is a frequent complication of diverse ischemic, nephrotoxic, and nephritic insults to the kidney all of which invariably produce obstruction of renal tubules by desquamated cells and debris. The pathogenetic role of tubular obstruction in the development of acute renal failure has been established in a series of microdissection studies (1, 2) and further advanced by servo-null pressure monitoring, which revealed an elevation of proximal tubular pressure after diverse renal insults (3–6). It has been found that after renal insults, the majority of desquamated cells are viable and contribute to the development of tubular obstruction (7).

These findings provided a rationale for our investigations into the stress-induced derangement of cell–matrix adhesion and facilitated cell–cell adhesion of renal tubular epithelial cells. Indeed, oxidant stress to renal epithelia resulted in redistribution of several subunits of integrin receptors from the predominantly basal location to the apical cell membrane (8). This phenomenon has underscored both the detachment of cells from the matrix and their potential cytoattractant properties (8, 9). The present study was undertaken to examine cytoattractive properties of stressed epithelial cells *in vitro* and *in vivo*, as well as the involvement of integrin receptors in this process. Given that many integrin receptors recognize the Arg-Gly-Asp (RGD) sequence presented by matrix proteins (10–13), the effect of synthetic RGD peptides on cytoattraction and on the dynamics of proximal tubular pressure was examined.

MATERIALS AND METHODS

Cell Culture. BSC-1 cells, an established line of African green monkey kidney cells, were routinely grown in minimal essential medium (MEM) supplemented with 10% newborn calf serum. These cells display a repertoire of integrin receptors similar to that found in the proximal tubular epithelium (8, 14) and crossreact with the antibodies produced against human integrin receptors (8). For cell–cell adhesion studies, BSC-1 cells were cultured in 24-well clusters precoated with fibronectin (Sigma) at 50 μg/ml or laminin (Collaborative Research) at 30 μg/ml. Suspensions of BSC-1 cells used in cell–cell adhesion assays were prepared by scraping of monolayers grown on uncoated plastic surfaces.

Cell–Cell Adhesion Assay. BSC-1 cells in suspension were loaded with a vital fluorescence indicator, 10 μM calcein acetoxymethyl ester (Molecular Probes) during a 45-min incubation in Krebs–Henseleit buffer [111 mM NaCl/5.4 mM KCl/20 mM $NaHCO_3$/1.8 mM $CaCl_2$/0.8 mM $MgSO_4$/1.0 mM Na_2HPO_4/10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) gassed with 5% CO_2 in air, adjusted to pH 7.4, and supplemented with 0.1% glucose and 0.1% bovine serum albumin]. Only viable cells are capable of accumulating calcein within the cytoplasm. Excess fluorescent indicator was removed by repeated centrifugation/resuspension in the same buffer, followed by cytometry, adjustment of cell density to $\approx 5 \times 10^5$ per ml, and addition of $\approx 1.1 \times 10^5$ cells per well to the monolayers grown in 24-well clusters. Such cell density equaled the number of monolayer cells per well, resulting in a ratio of adherent to suspended cells of about 1. Cells were coincubated at 37°C in an atmosphere of 5% CO_2/95% air for 30–120 min (standard duration was 60 min, at which point adhesion was linearly related to incubation time) with occasional rocking. On completion of this step, incubation medium was aspirated, and each well was gently washed with 2 ml of the same medium to remove nonadherent cells. BSC-1 cells were lysed in 1 ml of distilled water by a freezing–thawing cycle, and fluorescence per well was measured with a Perkin–Elmer fluorometer. Alternatively, nonlysed cell monolayers were studied by fluorescence microscopy and attached calcein-loaded cells were counted and expressed as a percentage of cells in the field. Experiments with coincubation of monolayers and suspended BSC-1 cells were performed in the presence of 0.5 mM hexapeptide GRGDNP or GRGESP, used as inactive control (Telious Pharmaceuticals, San Diego). Experiments were performed with intact BSC-1 mono-

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Abbreviation: $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration.

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layers and with monolayers pretreated with 1 mM H_2O_2 for 10 min, which results in apical expression of integrin receptors (8).

Preparation of Coated Beads. Adhesion of latex beads to BSC-1 cell monolayers was studied in a separate series of experiments utilizing coated beads. Polystyrene latex beads 1.02 μm in diameter were obtained from Sigma. Prior to experiments, 50 μl of beads was added to the same volume of the Krebs–Henseleit buffer, followed by 20 μl of bovine serum albumin (20 mg/ml). After 10 min of incubation, 50 μl of laminin (1 mg/ml) or fibronectin (1 mg/ml) was added for an additional 10 min, as described previously (15). The preparation was sonicated for 5 sec at 60 W and the thus-prepared mixture of $albumin_{in}$/$laminin_{out}$ (or $fibronectin_{out}$) latex beads was immediately used in experiments assessing the functional competence of apically expressed integrin receptors. In control experiments, latex beads were prepared as described, but with initial laminin coating, followed by albumin coating (15, 16). In studies on adhesion of beads to BSC-1 monolayers, fluorescent beads of 5.6-μm diameter were used (Flowcytometry Standards, Research Triangle Park, NC).

Monitoring of Cytosolic Ca^{2+} Concentration ($[Ca^{2+}]_i$). BSC-1 cells were grown on glass coverslips precoated with fibronectin or laminin, as described above. At confluence, cells deprived of serum for 24 hr were loaded with 2 μM fura-2 acetoxymethyl ester (Molecular Probes) for 45–60 min, washed in the Krebs–Henseleit buffer, and studied microspectrofluorometrically with a Photon Technology International Delta-scan II (Princeton, NJ) and an inverted Nikon Diaphot microscope equipped with quartz optics. Measurements of $[Ca^{2+}]_i$ were performed as described (17, 18) and calibrated according to the technique of Williams *et al.* (19). Alternatively, fura-2 fluorescence was monitored with a digital ratio image-analysis system equipped with imaging software (Universal Imaging, Media, PA), as previously detailed (17). Coverslips were mounted in a temperature-controlled Leiden chamber (Medical Systems, Greenvale, NY) and continuously perfused with the laminin-, fibronectin-, or BSA-coated latex beads. Experiments were performed with intact BSC-1 cell monolayers and with cells exposed to 1 mM H_2O_2 for 10 min (this treatment results in reorientation of integrin receptor subunits).

Monitoring of Proximal Tubular Pressure. Nineteen male Sprague–Dawley rats weighing 250–300 g were anesthetized with intraperitoneal injection of pentobarbital (50 mg/kg of body weight) and prepared for experiments by inserting catheters in the carotid artery (blood pressure monitoring), jugular vein (infusion of 0.9% NaCl at 50 μl/min), and femoral vein (injection of lissamine green solution to identify tubular lumens). The left kidney was then isolated and placed in a micropuncture cup, and a 30-min recovery period was allowed. Control measurements of proximal tubular pressure (3) were performed in five to eight tubules; then 50 units of heparin in 1 ml of 0.9% NaCl was infused intravenously and the left renal artery was clamped for 60 min. After release of the clamp, vehicle (0.9% NaCl) or synthetic peptide (10 mM solution in 0.9% NaCl) was infused into the left renal artery at 20 μl/min for 5 hr. Measurements of tubular pressure were performed every hour in five to eight tubules. Multiple samples of tubular pressure were averaged to give a single value for each rat at each time point. Experimental results were analyzed by analysis of variance for replicate measurements and by Scheffe test. $P < 0.05$ was considered a significant difference between groups.

RESULTS

Recent work (8) has demonstrated that oxidant stress to BSC-1 cells results in the reorientation of the α_3 integrin subunits from the predominantly basolateral location to the apical cell surface. Such an ectopic apical expression of integrin subunits was encountered in 100 of 195 stressed cells and in only 25 of 185 control cells (8). To test the functional competence of integrin receptors expressed on the apical surface of stressed cells, several approaches were used. First, we tested the possibility that these apically expressed integrin receptors are capable of mediating cell–cell adhesion. Adherent stressed cells displayed a significant increase over the nonstressed monolayers in cytoattraction of BSC-1 cell suspension (Fig. 1). When cell–cell adhesion assays were performed in the presence of the RGD-containing peptide, adhesion of suspended BSC-1 cells to intact monolayers was not affected, whereas adhesion to the stressed cells was significantly inhibited (Fig. 1).

Next, the functional competence of integrin receptors expressed ectopically on the apical cell surface was evaluated by using polystyrene latex beads coated with laminin or fibronectin. BSC-1 cells were loaded with the acetoxymethyl ester of the fluorescent Ca^{2+} indicator fura-2, and $[Ca^{2+}]_i$ was monitored by digital ratio image analysis as described in *Materials and Methods*. Stressed BSC-1 cells, but not intact BSC-1 cells, responded to the perfusion of laminin-coated latex beads with an increase in $[Ca^{2+}]_i$ (Fig. 2). This phenomenon did not occur when perfusate contained bovine serum albumin-coated latex beads, indicating that the observed increase in $[Ca^{2+}]_i$ was mediated by the specific engagement of apically expressed integrin receptors. Similar results were obtained with fibronectin-coated latex beads (data not shown). These results indicated that the ectopic apical expression of integrins is a prerequisite for cell activation by ligands attached to a solid phase.

Use of fluorescent laminin-coated latex beads in the cell adhesion assay revealed that their adhesion to the stressed BSC-1 cells was significantly enhanced compared with un-

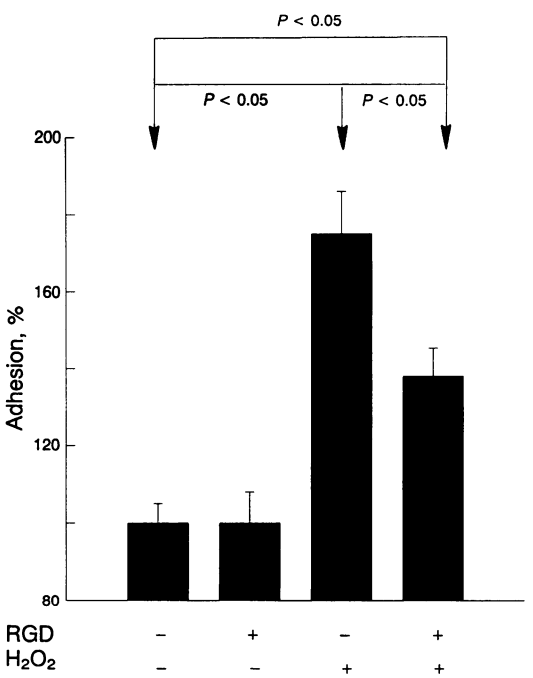

FIG. 1. Cytoattractant properties of stressed BSC-1 cells are counteracted by the RGD-containing peptide GRGDNP. Cell–cell adhesion assays ($n = 4$ in each group) were performed as described in *Materials and Methods*. Adhesion of BSC-1 cells to intact monolayers was arbitrarily taken as a unit, and other test conditions were compared with this standard. Note that oxidant stress almost doubled cell–cell adhesion and that this effect was inhibited by 0.5 mM GRGDNP. The peptide did not affect cell adhesion to intact cell monolayers and did not affect adhesion of intact BSC-1 cells.

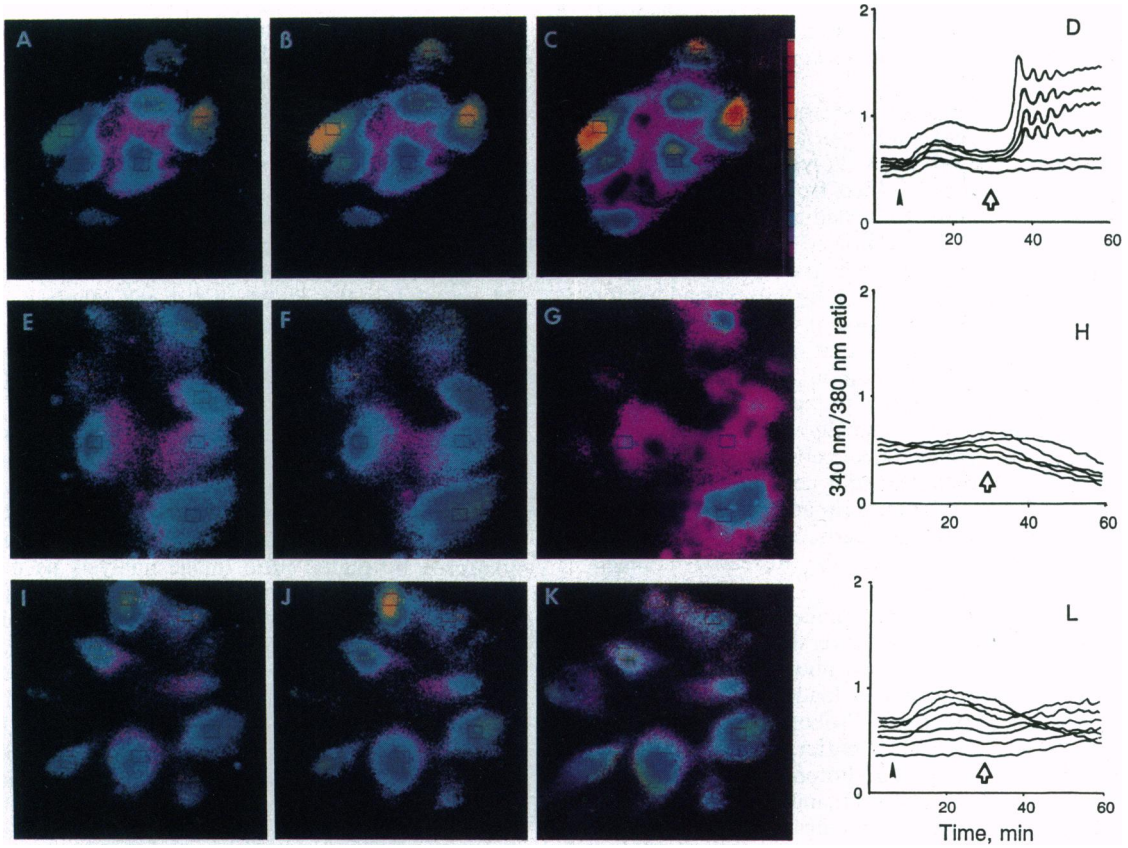

FIG. 2. Quantitative image analysis of fura-2 fluorescence in BSC-1 cells stimulated with laminin-coated latex beads. Pseudocolor images of 340 nm/380 nm ratio fluorescence intensity of BSC-1 cells exposed to 1 mM H_2O_2 followed by laminin-coated latex beads (*Top*) or albumin-coated latex beads (*Bottom*) and intact BSC-1 cells exposed to laminin-coated latex beads (*Middle*). Pseudocolor scale is shown at right in *C*. *D*, *H*, and *L* represent real-time 340 nm/380 nm ratio continuously recorded from individual cells throughout the experiments (tracings were manually copied from the computer screen). Arrowheads denote the addition of H_2O_2, and open arrows denote the addition of latex beads. Latex beads sedimented on the surface of monolayers in all experiments; however, slowly oscillating increase in fura-2 fluorescence was observed only with laminin-coated latex beads (compare *A–C* with *I–K*) applied only to prestressed monolayers (compare *A–C* with *E–G*). Addition of 1 mM H_2O_2 *per se* caused a small and transient increase in $[Ca^{2+}]$. (×400.)

stimulated monolayers (Fig. 3). In contrast, albumin-coated beads did not demonstrate similar preferential binding. GRGDNP (0.5 mM) virtually abolished the increase in adhesion of laminin-coated beads but did not affect the adhesion of albumin-coated beads. These data indicate that apically expressed ectopic integrins are competent in recognition and binding of RGD-containing matrix proteins attached to a solid phase.

If analogous reorientation of functionally competent integrin receptors occurs *in situ* during development of acute renal failure, this may result in inappropriate interactions of such apically expressed receptors with matrix proteins or other integrin receptors present on the surface of desquamated renal tubular epithelial cells. To test this possibility, experiments were performed with rats subjected to 60 min of unilateral renal ischemia. Changes of proximal tubular pressure prior to ischemia and following the release of renal artery occlusion were monitored with a servo-null pressure device and proximal tubule micropuncture, as detailed in *Materials and Methods*. Monitoring of proximal tubular pressure in rats following release of a 60-min clamp of the renal artery confirmed previous observations that this model of acute renal failure results in development of tubular obstruction (3–6). One hour after release of the arterial clamp, proximal tubular pressure increased from 12.3 ± 0.8 to 21.9 ± 3.2 mm Hg (1 mm Hg = 133 Pa), thus directly demonstrating an early involvement of tubulo-obstructive phenomena in the pathogenesis of acute renal failure (Fig. 4). The increment in proximal tubular pressure was most prominent 2–3 hr after release of the renal artery clamp, followed by a gradual recovery of tubular pressure. When the synthetic RGD-containing peptide GRGDNP (instead of 0.9% NaCl) was infused into the renal artery during the postischemic period, the ischemia-induced increase in proximal tubular pressure was virtually abolished. This effect was specific for the RGD-recognizing integrin receptors, since the inactive peptide GRGESP did not modify proximal tubular pressure responses to 60 min of renal ischemia.

DISCUSSION

Clinical studies in patients with acute renal failure have established an important phenomenon, an increased urinary excretion of viable tubular epithelial cells (7). These cells might be responsible for the tubular obstruction, increased proximal tubular pressure, and temporary cessation of glomerular filtration (1, 2, 7, 9) that are characteristic of acute renal failure. On the basis of these findings, it seemed promising to evaluate the possible role of focal adhesions in the development of epithelial cell desquamation.

Previous work demonstrated that exposure of BSC-1 cells to oxidant stress resulted in the loss of focal contacts, redistribution of the α_3 subunits of integrin receptors from the basal cell surface to the apical membrane, and eventual cell detachment from the matrix without significant loss of viability (8). It should be noted that oxidant stress represents a common pathogenetic denominator for diverse ischemic and nephrotoxic causes of acute renal failure (20–22), thus pro-

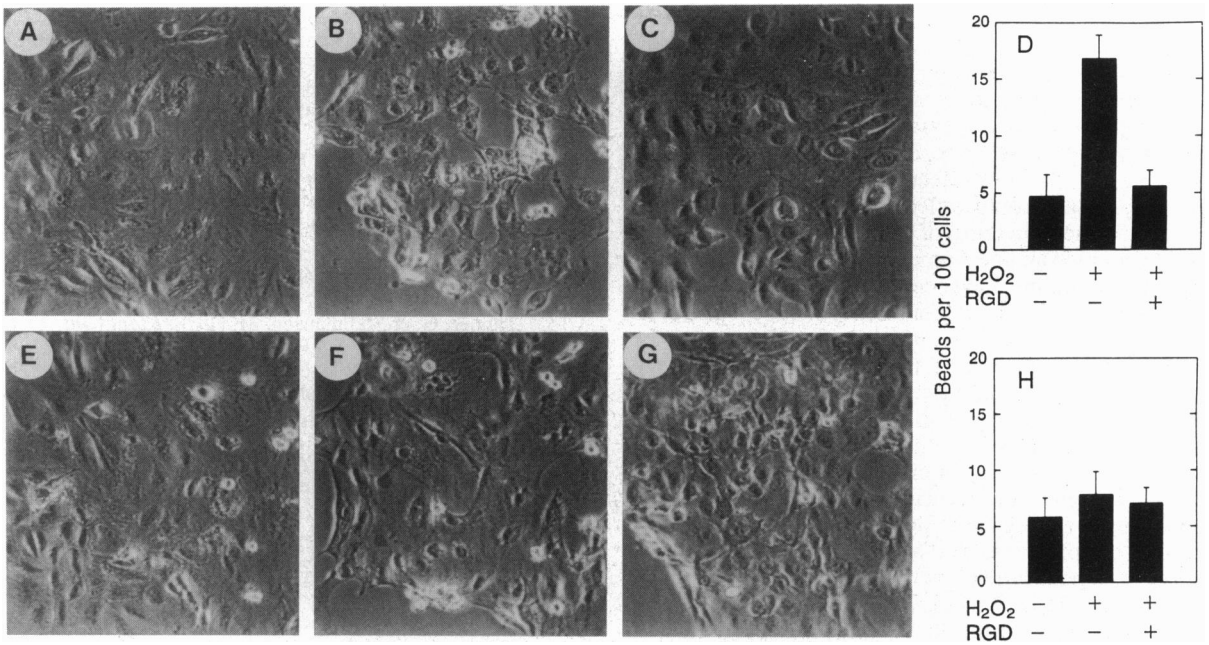

FIG. 3. Adhesion of laminin-coated (*Upper*) or albumin-coated (*Lower*) fluorescent latex beads to BSC-1 cells. Combined fluorescence/brightfield micrographs are representative of the following experimental conditions: control (*A* and *E*); 1 mM H_2O_2 for 10 min (*B* and *F*); 1 mM H_2O_2 for 10 min followed by 0.5 mM GRGDNP (*C* and *G*). Number of fluorescent beads that adhered within 60 min to the monolayers was calculated and expressed per 100 BSC-1 cells (*D* and *H*). Note that oxidant stress resulted in an increased adhesion of laminin-coated, but not albumin-coated, fluorescent beads and that GRGDNP virtually abolished the increase in adhesion of laminin-coated beads without effect on adhesion of albumin-coated beads. (×200.)

viding a valuable tool to investigate cellular responses to insult. Because integrin receptors undergo reorientation after this insult (8), we hypothesized the following scenario for the development of tubular obstruction. The loss of basolateral expression of integrins may be responsible for the detachment of cells from the matrix, whereas expression of integrin receptors on the apical surface of tubular epithelial cells could account for facilitated adhesion of desquamated cells and eventual tubular obstruction (9). The key question of the present study was to examine the competence of apically expressed integrin receptors.

This question was addressed in both *in vitro* and *in vivo* experiments bringing about two major sets of complementary findings. First, stressed BSC-1 cell monolayers displayed cytoattractive properties toward suspended BSC-1 cells and this augmentation in cell–cell adhesion was specifically inhibited by the synthetic RGD peptide. Second, this finding was corroborated by the whole-animal experiments demon-

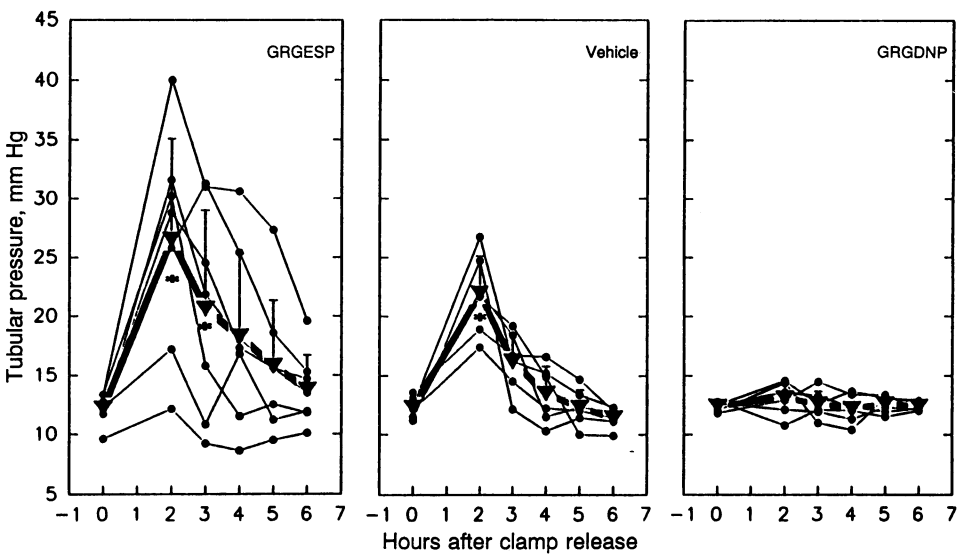

FIG. 4. Ischemia-induced increase in proximal tubular pressure is attenuated by the peptide GRGDNP. Proximal tubular pressure was monitored before and 2–6 hr after 60 min of renal artery occlusion, as detailed in *Materials and Methods*, in rats receiving renal arterial infusion of inactive synthetic peptide (GRGESP, $n = 7$), vehicle (NaCl, $n = 6$), or RGD-containing synthetic peptide (GRGDNP, $n = 6$). Each point represents a mean of five to eight separate measurements performed in a single animal. Results obtained from the same rat are connected to demonstrate the time course of changes in proximal tubular pressure in individual rats. Filled triangles connected with thick lines depict mean ± SD for each group; asterisks represent $P < 0.05$ vs. preischemic control period (control, time zero). Note that GRGDNP infusion virtually abolished the postischemic increase in proximal tubular pressure.

strating that the synthetic RGD sequence prevented the ischemia-induced increase in proximal tubular pressure. Since this parameter represents a direct measure of the degree of tubular obstruction, it is conceivable that the observed ischemia-induced increase in the proximal tubular pressure is governed by RGD-recognizing integrin receptors. Alternatively, the observed effect of RGD peptide on proximal tubular pressure could reflect an increase in vasoconstriction and further lowering of glomerular capillary pressure. However, such an alternative explanation seems highly unlikely because inulin clearance in this ischemic model is reduced by 94–97% from the control level (4, 5). One can argue that the effect of RGD peptide was instead due to the enhanced desquamation of tubular epithelial cells and to the subsequent leak of the ultrafiltrate from the lumen to the interstitium. This does not seem to be the case, however, because in this time frame an ≈50-fold higher concentration of the GRGDNP did not affect cell–matrix adhesion *in vitro*. Further, the presence of heparin in the renal circulation before and during the ischemic period make it unlikely that the effect of the peptide was mediated by inhibition of platelet aggregation. It remains unclear whether cell–cell adhesion in the *in vivo* model occurs mainly among desquamated cells (analogous to the aggregation) or between the desquamated and *in situ* cells. These uncertainties notwithstanding, the most important property of cell–cell adhesion in acute renal failure is represented by its blockade with the peptide GRGDNP. This fact supports the notion that integrins play a crucial role in the pathogenesis of tubular obstruction in acute renal failure.

In conclusion, this study provides strong evidence of functional competence of apically expressed integrin receptors in stressed BSC-1 cells, the role of RGD-recognizing integrin receptors in cell–cell adhesion *in vitro*, and their role in acute ischemic renal failure *in vivo*. Engagement of the RGD-recognizing integrin receptors in the development of tubular obstruction during the course of renal failure opens a perspective for preventive therapy.

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