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Anti-Inflammatory Effects of α_v Integrin Antagonism in Acute Kidney Allograft Rejection

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Integrin-mediated cell adhesion and signaling is essential to vascular development and inflammatory processes. Elevated expression of integrin $\alpha_v\beta_3$ has been detected in ischemia-reperfusion injury and rejecting heart allografts. We thus hypothesized that the inhibition of α_v -associated integrins may have potent anti-inflammatory effects in acute kidney allograft rejection. We studied the effects of a peptidomimetic antagonist of α_v integrins in two rat models of renal allotransplantation, differing in degree of major histocompatibility complex mismatch. Integrin $\alpha_v\beta_3$ was up-regulated in rejecting renal allografts. Integrin antagonist reduced the histological signs of acute rejection, the intensity of the mononuclear cell infiltration, and cell proliferation in the grafted kidneys. This could be correlated to a reduced leukocyte-endothelial interaction and an improved peritubular microcirculation observed by intravital microscopy. *In vitro* under laminar flow conditions, the arrest of monocytes to interleukin-1 β -activated endothelium was decreased. Furthermore, in co-culture models the proliferation and transmigration of monocytes/macrophages, endothelium, and fibroblasts induced by renal tubular epithelia was efficiently inhibited by α_v integrin antagonism. These data reveal an important role of this integrin subclass in leukocyte recruitment and development and maintenance of

acute rejection; blockade of α_v integrins may provide a new therapeutic strategy to attenuate acute allograft rejection. (*Am J Pathol* 2007, 171:1127–1139; DOI: 10.2353/ajpath.2007.070329)

Adhesion molecules such as integrins are involved in multiple stages of immune and inflammatory responses; they are implicated in antigen presentation and in the extravasation and migration of leukocytes through the extracellular matrix.^{1,2} Integrins are a family of glycosylated, heterodimeric transmembrane adhesion receptors that consist of noncovalently bound α and β subunits. They have both structural and regulatory functions, building strong adhesive bridges between cells or between cells and extracellular matrix as well as providing bidirectional transmission of signals across plasma membranes.^{3,4}

α_v Integrins form a subfamily of five integrins ($\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$, and $\alpha_v\beta_8$) that recognize a group of overlapping ligands, which generally contain the canonical tripeptide recognition sequence arginine-glycine-aspartic acid (RGD). This subfamily of integrins seems to play important and specific roles in a number of cellular processes, including proliferation, differentiation, survival, and migration; they influence growth and permeability of blood vessels^{5–7}; and several of these processes can be found in allograft rejection. However, little is known about the function of α_v integrins in renal transplantation.

There are correlative studies suggesting that the $\alpha_v\beta_3$ integrin could be of importance in allograft rejection. The cells that express high levels of $\alpha_v\beta_3$ include activated macrophages and endothelial cells.^{4,8,9} Endothelial cells are primary targets in allograft rejection; macrophages

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represent a major mononuclear cell population in rejection.¹⁰ Integrin $\alpha_v\beta_3$ has also been found in neutrophils, vascular smooth muscle cells, mesangial cells, tubular epithelial cells, and fibroblasts.^{8,11} Its expression is modulated by several cytokines [eg, interleukin (IL)-4, tumor necrosis factor- α] and growth factors (eg, platelet-derived growth factor, fibroblast growth factor). $\alpha_v\beta_3$ itself can interact and modulate the expression of growth factors/growth factor receptors (eg, platelet-derived growth factor, vascular endothelial growth factor receptor-1) and release of cytokines (eg, tumor necrosis factor- α , interferon- γ) in different cell types,^{12–14} and elevated levels of these mediators have been detected in transplanted organs.^{15,16} Furthermore, ligands of $\alpha_v\beta_3$ integrin include osteopontin, fibronectin, fibrinogen, thrombospondin, von Willebrand factor, collagen, and matrix metalloproteinase (MMP)-2, which are also present in rejecting transplanted organs.^{17–19}

The recruitment of leukocytes from the blood into tissue is essential for the development and maintenance of inflammation in acute rejection and is regulated by a complex network of cytokines, chemokines, and adhesion molecules.^{12,20} $\alpha_v\beta_3$ was found to promote monocyte migration, to be involved in neutrophil mobility on extracellular matrix substrates *in vitro*, and leukocyte migration across the mesenteric endothelium *in vivo*.^{21,22} Moreover, leukocyte infiltration in transplanted kidneys is associated with tubular epithelial cell and interstitial fibroblast activation and proliferation perpetuating acute inflammation and initiating chronic changes.²³ The up-regulation of tubular α_v integrin was found to be associated with the expression of other adhesion molecules, infiltration of macrophage, and the presence of markers of disease progression in human glomerulonephritis.²⁴ Increased expression of $\alpha_v\beta_3$ has been found in acute cellular rejection of human heart transplants and transplant coronary vasculopathy as well as in ischemia-reperfusion injury of rat kidneys; its function was not investigated in these settings.^{25–27}

We hypothesized that the antagonism of α_v integrins could inhibit cell recruitment across activated microcirculatory endothelia, thereby limiting rejection phenomena in kidney allografts. The effects of S247, a peptidomimetic RGD-based α_v integrin antagonist, were investigated in two models of allotransplantation with different major histocompatibility complex disparity; expression of $\alpha_v\beta_3$ in kidney allografts was determined; the effects on leukocyte-endothelial interactions (LEIs) were studied *in vivo* by intravital microscopy and *in vitro* by a laminar flow system. Because of the close proximity of tubular epithelial cells to monocytes, peritubular capillary endothelial cells, and fibroblasts in interstitium of kidney graft co-culture models were established to study the role of α_v integrin signaling on transmigration and proliferation of these cells in the renal interstitium. We have now shown that this integrin subclass is important for development of inflammatory infiltrates into the allograft. Firm attachment of mononuclear cells to the vasculature of the graft and their extravasation from vessels into nearby tissue, as well as endothelial

and fibroblast proliferation and migration, were reduced by S247. Blockade of α_v integrins led to inhibition of acute rejection of kidney allografts.

Materials and Methods

Reagents and Cell Culture

Primary isolated human dermal fibroblasts (HDFs) and human dermal microvascular endothelial cells (HDMECs) (Promocell, Heidelberg, Germany) were maintained in culture as described previously.^{28,29} The immortalized human endothelial cell line (HMEC-1; Tumorbank, DKFZ, Heidelberg, Germany) was grown in endothelial cell medium (endothelial cell growth medium MV; Promocell). For the immortalized human proximal tubular cell line HK-2 (American Type Culture Collection, Manassas, VA), serum-free media (K-SFM; Gibco Life Technologies, Karlsruhe, Germany) were supplemented with 5 ng/ml human recombinant epidermal growth factor and 0.05 μ g/ml bovine pituitary extract. The human monocytic cell line THP1 was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% penicillin/streptomycin, 1% glutamine, and 1% HEPES (Sigma-Aldrich, Deisenhofen, Germany). For macrophage differentiation of THP1 cells, 160 nmol/L phorbol-12-myristate-13-acetate (Sigma-Aldrich) was added for 72 hours. Human IL-1 β was purchased from Peprotech (London, UK).

S247 is a synthetic low-molecular-weight peptidomimetic ligand with an amino acid sequence RGD; this inhibits the activities of the α_v subunit-containing integrins with a relative specificity for the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ at nanomolar concentrations and significantly less antagonistic potency against two other integrins that also use the RGD sequence motif for ligand recognition, $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$. S247 is >3 orders of magnitude more selective for $\alpha_v\beta_3$ than for $\alpha_{IIb}\beta_3$.³⁰ S247 was generously provided by Pharmacia Corporation (Oncology Pharmacology, Discovery Research, Pharmacia Corporation, St. Louis, MO).

Animals and Renal Transplantation

Inbred male rats purchased from Charles River Laboratories GmbH (Sulzfeld, Germany) were used for all transplant experiments. In the first model, Fisher 344 rats [F344 RT1^{lv}; body weight (bw), 230 to 250 g] served as donors for kidney transplants and Brown-Norway rats (BN RT1ⁿ; bw, 180 to 200 g) were used in the second model. Lewis rats (LEW RT1^l; bw, 200 to 230 g) served as recipients. For adjustment of ureter diameters, rats of different body weights were chosen. All animals were fed with standard rat chow (Altromin, Lage, Germany) and had free access to tap water. All experiments were in accordance with the German legislation on the protection of animals.

Renal transplantation was performed by a technique modified from Lee³¹ as previously described. In the Fisher 344 to Lewis (F344-LEW) model, the right kidney of the recipient was left in place to enhance allograft rejection.

tion³² and to serve as a control for the effect of S247. In the BN-LEW model, nephrectomy was performed bilaterally. Ice-cold ischemic time of the donor kidney varied between 40 minutes (F344-LEW model) and 10 minutes (BN-LEW model); warm ischemia was ~20 minutes in every group.

Experimental Protocol and Experimental Groups

S247 was dissolved in water diluted to 0.9% sodium chloride and injected subcutaneously twice a day at a dose of 25 mg/kg bw in all treated animals. In addition, in BN-LEW allografts cyclosporine A (CsA; kindly provided by Novartis, Basel, Switzerland), dissolved in olive oil, was administered subcutaneously once per day at a dose of 1.25 mg/kg bw.

The experimental groups were as follows: group 1: F344 kidneys into LEW rats with one endogenous kidney; group 1a: without S247 for 10 days ($n = 9$); group 1b: with S247 at 50 mg/kg bw per day for 10 days ($n = 8$); group 2: F344 kidneys into LEW rats with one endogenous kidney for *in vivo* microscopy; group 2a: without S247 for 10 days ($n = 8$); group 2b: with S247 at 50 mg/kg bw per day for 10 days ($n = 8$); group 3: sham-operated (nontransplanted) F344 rats ($n = 7$); group 4: BN kidneys into bilaterally nephrectomized LEW rats with CsA 1.25 mg/kg bw per day; group 4a: without S247 for 7 days ($n = 8$); and group 4b: with S247 at 50 mg/kg bw per day for 7 days ($n = 8$).

Measurement of Graft Functional Parameters and Systolic Arterial Pressure

For measurements of serum creatinine and proteinuria, rats were kept in metabolic cages 24 hours before the end of the experiment. Blood and urine samples were analyzed by a Hitachi 9-17 E autoanalyzer (Hitachi, Frankfurt/M, Germany). Systolic blood pressure was determined before sacrifice by tail-cuff plethysmography under light ether anesthesia.³³

Histological Analysis

Renal allografts were removed in deep anesthesia, quickly blotted free of blood, weighed, and processed as required for histology and immunohistology. For histology, the kidneys were cut into 1-mm slices and either immersion-fixed in 4% formaldehyde in phosphate-buffered saline (pH 7.35) (99 mmol/L NaH_2PO_4 , 108 mmol/L NaH_2PO_4 , and 248 mmol/L NaCl) for 24 hours at 4°C or fixed in methacarn (60% methanol, 30% chloroform, and 10% acetic acid) for 8 hours and then embedded in paraffin.

Renal Morphological Studies

Light microscopy was performed on 3- μm sections stained by periodic acid-Schiff. Kidneys were evaluated for evidence of vascular, glomerular, and tubulointerstitial damage and scored as previously described.^{34,35}

Immunohistochemistry

Immunohistochemical staining was performed on 3- μm sections of paraffin-embedded tissue except for $\alpha_v\beta_3$, which was labeled on frozen sections of kidney grafts using a mouse monoclonal antibody from Biozol (Eching, Germany), detected by a tyramide signal amplification fluorescein technique (NEN kit; Life Science, Boston, MA). Mouse anti-rat monoclonal antibodies against ED1 (Serotec, Oxford, UK) for monocytes/macrophages in methacarn-fixed tissue, CD8 (Serotec) for cytotoxic T cells, and nuclear antigen Ki-67 (clone MIP 5; Dianova, Hamburg, Germany) for the detection of proliferating cells were used. Fibroblast subpopulations were identified by antibodies against vimentin (Progen, Heidelberg, Germany) and α -smooth muscle actin (α -SMA; Sigma-Aldrich). Formaldehyde-fixed tissues were microwave-treated. An alkaline phosphatase anti-alkaline phosphatase detection system was applied (Dako Cytomation A/S, Glostrup, Denmark). Glomerular-positive cells were counted in 50 glomeruli and given as the mean number per glomerular cross section. Interstitial-positive cells were counted in 20 high-power fields of the cortex, and the outer stripe of the outer medulla and assessed as mean per high-power field. Controls omitting the first or the second antibody for each section were tested negative.

Intravital Microscopy

Intravital microscopy was performed under ether anesthesia. First, the left common carotid artery was cannulated with a flexible polyethylene tube (Dow Corning, Newark, NJ) and attached to a pressure sensor (Statham, Oxnard, CA) for continuous blood pressure monitoring, injection of fluorescent dyes, and volume replacement. Body temperature was maintained by a heating device and monitored with a rectal probe. The abdomen was opened by a longitudinal midline incision, and the capsule of the kidney removed. The kidney was then placed on a specially constructed stage to avoid respiratory artifacts. A coverglass that was held by capillary forces of the surrounding Ringer solution was placed on the renal surface and attached to the stage by elastic silicone glue. Compression of the kidney did not occur. During microscopy, the kidney was continuously rinsed with warm (37°C) Ringer solution. After a stabilization period of 15 minutes, an intra-arterial injection of 0.6 ml of 0.5% fluorescein isothiocyanate-labeled dextran (500.000 D; Sigma-Aldrich) and 0.2 ml of 0.1% rhodamine-6G (Sigma-Aldrich) was used to visualize renal cortical microcirculation and circulating leukocytes, respectively. Anaphylactic reactions (hypotension and limb edema) after the injection of the dyes were not observed.

A Zeiss Axiotech vario 100 microscope for epi-illumination was used with a 100-W HBO mercury lamp (Carl Zeiss AG, Göttingen, Germany). Magnifications of $\times 200$ and $\times 400$ were achieved with $\times 20$ and $\times 40$ water immersion objectives. At least 10 sites of interest per animal were randomly selected for each objective, recorded by

means of a charge-coupled device video camera (Kappa Opto-Electronics GmbH, Gleichen, Germany) and transferred to a video system.

Analysis of microcirculatory parameters in the renal cortex of the kidney was performed off-line from videotape with an image analysis system (CapImage; Zeintl, Heidelberg, Germany). Functional capillary density was defined as the total length of perfused capillaries per cm^{-1} .³⁶ Red and white blood cell velocities were quantified with the line-to-shift diagram method in mm/second .³⁶ Furthermore, by using the fluorescent marker rhodamine-6G, LEIs were analyzed: rolling leukocytes were defined as a population of cells temporarily interacting with the vessel wall and thus having a velocity of at least 50% less than white blood cell velocity in the same vessel and counted throughout a period of 60 seconds; adherent leukocytes were defined as cells stationary for at least 30 seconds, and they are given as cells/mm^2 of vessel

wall surface assuming a cylindrical anatomy; transmigrated leukocytes were defined as cells that were visible outside the capillary vessel and counted after 60 minutes as number of cells/mm^2 of tubulointerstitial tissue.

In Vitro Flow System of Monocyte Recruitment to Microvascular Endothelium under Physiological Flow Conditions

The interaction of THP1 monocytes with endothelium (HMEC-1) was studied in laminar flow assays at $1.5 \text{ dyn}/\text{cm}^2$ after stimulation with IL-1 β (10 ng/ml) for 12 hours as described previously.³⁷ For inhibition experiments, monocytes and HMEC-1 cells were preincubated with S247 at different concentrations (0.1 to $0.5 \mu\text{mol}/\text{L}$) for 60 minutes. After 5 minutes of equilibration, rolling and firmly adherent leukocytes were quantitated in at least five optical fields per

Figure 1. A–D: Expression of $\alpha_v\beta_3$ integrin. The expression of $\alpha_v\beta_3$ in normal F344 (A and C) and allogeneic (B and D) (F344 to LEW) kidney grafts 10 days after transplantation; confocal light microscopy/immunofluorescence. A and C: Constitutive expression of $\alpha_v\beta_3$ in the perivascular area of a preglomerular artery, tubulointerstitium, specifically peritubular capillaries (asterisks) of a native F344 rat kidney. Increased expression of $\alpha_v\beta_3$ in untreated allografts: a preglomerular artery presenting $\alpha_v\beta_3$ expression at the endothelial surface (arrowheads) and media (M) as well as in the perivascular cellular infiltrate (arrows) (B and D); tubulointerstitium with increased $\alpha_v\beta_3$ expression in peritubular capillaries (asterisks) and expression in tubular epithelial cells in comparison to normal kidney (A and C). M, media; L, lumen. Original magnifications, $\times 200$.

experiment by analysis of images recorded with a long integration JVC 3CCD video camera and a JVC DR-M10 DVD recorder (JVC Deutschland GmbH, Friedberg, Germany). Rolling leukocytes (primary interaction) were defined as a population of cells temporarily interacting with the vessel wall and thus having a velocity at least 50% less than flow velocity and counted as number of cells throughout a period of 30 seconds. The number of firmly adherent cells (adherence >30 seconds) was quantitated per observation field (objective $\times 20$). Five independent experiments were done.

Co-Culture Experiments for Proliferation and Transmigration

In vitro co-culture experiments were performed as described previously with minor modifications.^{28,29} In brief, using a transwell model (Becton-Dickinson, Heidelberg, Germany), HDMEC and fibroblast (HDF) proliferation were assessed in the upper compartment in the absence or presence of HK-2 cells in the lower compartment with or without S247 treatment. HK-2 cells were grown in 24-well plates (lower compartment). Transwell inserts with a 0.4- μ m pore size (upper compartment) were plated with 20,000 HDMECs or HDFs. For the S247 groups, fibroblasts and endothelial cells were preincubated for 1 hour with 1 μ mol/L S247. After S247 treatment transwells were transferred into the 24-well plates, incubated for 72 hours at standard conditions before counting the fibroblasts and endothelial cells.

For co-culture transmigration assay, Matrigel-coated (0.78 mg/ml) transwells with 8- μ m pore size (Becton-Dickinson) were used. HK2 cells were seeded in 24-well plates (bottom wells). Phorbol-12-myristate-13-acetate-induced macrophages (THP1 cells) or HDFs were added to the transwells (upper compartment) and preincubated with or without 1 μ mol/L S247 for 1 hour. The transwells were transferred in 24-well plates. After 18 hours of incubation, THP1 cells or fibroblasts that had invaded the underside of the membrane were fixed and stained with

Diff-Quick II solution (Dade Behring, Eschbom, Germany), sealed on slides and counted by microscopy (number of migrated cells per eight optical fields at $\times 40$ objective and $\times 10$ oculars). Experiments were performed at least in quadruplicates.

Statistical Analysis

All data are presented as mean $\pm$ SEM or mean $\pm$ SD. Data were analyzed by the two-sided nonparametric Mann-Whitney *U*-test. A *P* value of less than 0.05 was considered to show a significant difference between two groups.

Results

Expression of $\alpha_v\beta_3$ Integrin in Rejecting Kidney Allografts

By immunostaining constitutive expression of $\alpha_v\beta_3$ could be observed in peritubular capillaries of normal F344 rat kidneys (Figure 1, A and C). In comparison to F344 kidneys, the intensity of $\alpha_v\beta_3$ staining was increased in tubular epithelial cells (cytoplasm) and peritubular capillaries in allografts; a faint positive signal could be observed on the endothelial surface and in the media of preglomerular arteries. An important number of $\alpha_v\beta_3$ -expressing cells were present in the perivascular cellular infiltrates of the allografts (Figure 1, B and D). Kidney sections incubated with normal serum lacked any staining.

Effects of S247 on Acute Rejection, Mononuclear Cell Infiltration, and Cell Proliferation in Allografts

At day 10 after transplantation, signs of vascular (endothelialitis, transmural arteritis) and tubulointerstitial rejection (interstitial infiltration and tubulitis) were evident without immunosuppression in F344-LEW kidney allografts.

Table 1. Histological Analysis of the Effects of α_v Integrin Blockade on Acute Rejection in F344 to LEW Allografts; Summary of Immunohistochemical Analysis of Cellular Infiltrate and Proliferation

	Control	S247
Tubulointerstitial area		
Tubulointerstitial inflammation (score)	152.2 $\pm$ 15.1	85.0 $\pm$ 17.1*
ED 1 (cells/HPF)	116.4 $\pm$ 13.9	66.9 $\pm$ 7.3*
CD 8 (cells/HPF)	97.1 $\pm$ 7.9	51.7 $\pm$ 5.7***
Ki-67 (cells/HPF)	97.7 $\pm$ 9.8	39.6 $\pm$ 5.7***
Vimentin (cells/HPF)	93.6 $\pm$ 2.9	73.9 $\pm$ 5.8*
α -SMA (cells/HPF)	35.9 $\pm$ 2.9	18.5 $\pm$ 2.2***
Acute vascular injury (score)	92.2 $\pm$ 18.4	34.6 $\pm$ 5.9**
Glomeruli		
Acute glomerular injury (score)	18.4 $\pm$ 2.3	13.1 $\pm$ 3.6
ED 1 (cells/glomerular cross section)	5.9 $\pm$ 0.44	4.5 $\pm$ 0.34*
CD 8 (cells/glomerular cross section)	4.0 $\pm$ 0.30	2.3 $\pm$ 0.22***
Ki-67 (cells/glomerular cross section)	4.9 $\pm$ 0.29	3.2 $\pm$ 0.35**
Vimentin (cells/glomerular cross section)	3.4 $\pm$ 0.3	2.0 $\pm$ 0.3**
α -SMA (cells/glomerular cross section)	2.1 $\pm$ 0.4	1.3 $\pm$ 0.1

Significant differences between untreated (control) and α_v integrin antagonist (S247)-treated allografts.

P* < 0.05, *P* < 0.01, and ****P* < 0.001 versus control.

Figure 2. A–F: Histological signs of rejection. **A:** Vascular rejection with infiltration of the intima and media by mononuclear cells in an untreated transplant kidney. **B:** Preglomerular artery of a S247-treated animal with a few mononuclear cells adherent to the endothelium 10 days after transplantation (F344 to LEW). **C** and **E:** Renal transplants showing an increased number of glomerular and tubulointerstitial ED1-positive monocytes/macrophages (**C**) and α -SMA-positive fibroblast-like cells (**E**). **D** and **F:** In contrast, S247-treated animals had significantly less ED1-positive and α -SMA-positive cells. **A** and **B:** Periodic acid-Schiff staining; **C** and **D:** immunostaining for ED1; **E** and **F:** immunostaining for α -SMA. Original magnifications: $\times 200$ (**A** and **B**); $\times 400$ (**C–F**).

S247 administration resulted in a significant decrease of vascular injury and tubulointerstitial inflammation in comparison to the untreated animals (Table 1 and Figure 2, A and B). The mononuclear cell infiltrate, detected by ED1-

positive monocytes/macrophages and CD8-positive cytotoxic T lymphocytes, was significantly less in the tubulointerstitial compartment and glomeruli of S247 grafts (Table 1 and Figure 2, C and D) when compared with

Table 2. Histological Analysis of the Effects of α_v Integrin Blockade on Acute Rejection in BN to LEW Allografts; Summary of Immunohistochemical Analysis of Cellular Infiltrates and Proliferation

	Control	S247
Tubulointerstitial area		
Tubulointerstitial inflammation (score)	85.63 $\pm$ 12.97	32.50 $\pm$ 6.75***
ED 1 (cells/HPF)	131.10 $\pm$ 3.57	105.80 $\pm$ 9.86*
CD 8 (cells/HPF)	30.12 $\pm$ 1.07	15.29 $\pm$ 0.27***
Ki-67 (cells/HPF)	21.23 $\pm$ 4.51	20.84 $\pm$ 3.59
Vimentin (cells/HPF)	73.8 $\pm$ 3.8	63.7 $\pm$ 4.6
α -SMA (cells/HPF)	27.2 $\pm$ 2.3	9.7 $\pm$ 2.8***
Acute vascular injury (score)	67.02 $\pm$ 8.43	49.15 $\pm$ 7.91
Glomeruli		
Acute glomerular injury (score)	63.82 $\pm$ 8.62	39.14 $\pm$ 4.90*
ED 1 (cells/glomerular cross section)	15.73 $\pm$ 0.73	13.66 $\pm$ 1.02
CD 8 (cells/glomerular cross section)	2.44 $\pm$ 0.20	1.57 $\pm$ 0.14***
Ki-67 (cells/glomerular cross section)	3.88 $\pm$ 0.81	4.19 $\pm$ 0.77
Vimentin (cells/glomerular cross section)	5.2 $\pm$ 0.2	4.5 $\pm$ 0.4
α -SMA (cells/glomerular cross section)	0.8 $\pm$ 0.1	0.6 $\pm$ 0.1

Significant differences between untreated (control) and α_v integrin antagonist (S247)-treated allografts.* $P < 0.05$ and *** $P < 0.001$ versus control.

control grafts. Ten days after transplantation, a considerable number of fibroblasts/myofibroblasts (vimentin⁺ and α -SMA⁺) were present in the tubulointerstitium of kidney allografts. These cells were significantly decreased by the α_v integrin antagonist (Table 1 and Figure 2, E and F). Furthermore, the α_v integrin antagonist led to a reduced number of proliferating cells (Ki-67-positive) in the tubulointerstitium as well as in glomeruli of treated grafts ($P < 0.01$) (Table 1). Light microscopy showed no obvious effect of S247 on the endogenous contralateral kidney (data not shown). The strong histoincompatibility transplantation model BN to LEW, characterized by a predominant vascular rejection, leading to early graft loss without immunosuppression, was used to analyze the combined effects of α_v integrin antagonism with low-dose CsA (1.25 mg/kg bw per day) on acute rejection (day 7 after transplantation).

As presented in Table 2, S247 treatment in combination with CsA, ameliorated vascular rejection and significantly reduced glomerulitis and tubulointerstitial inflammation in comparison to treatment with CsA alone. By combined therapy, less CD8⁺ T lymphocytes were found in all compartments of kidney allografts, but the addition of S247 to CsA had no additional effect on infiltrating ED1⁺ monocytes/macrophages and proliferating cells in these grafts (Table 2).

Corresponding to a better graft morphology, serum creatinine was significantly lower in S247 animals in the BN-LEW model (Table 4). Proteinuria was significantly decreased by S247 treatment in both models (Tables 3

and 4). There were no differences in body weight and relative kidney weights in different groups. Systolic arterial blood pressure measured by tail plethysmography was in the normal range in all groups (Tables 3 and 4).

Effects of S247 on Microvascular Perfusion in Vivo in F344-LEW Kidney Allografts

To characterize the underlying processes involved in the reduction of acute rejection by the α_v integrin antagonist, we performed a microcirculatory analysis of peritubular capillaries by intravital microscopy. In the sham-operated kidneys, no alterations of renal perfusion of peritubular capillaries were observed as described by functional capillary density and red and white blood cell velocity. In contrast, in untreated F344-LEW allografts at day 10 after transplantation, a significant reduced length of perfused capillaries (functional capillary density) was observed, and red blood cell velocity and white blood cell velocity were decreased ($\Delta 50\%$). In addition, swelling of endothelial cells was seen in transplanted allografts. In contrast, S247 administration resulted in a significant increase of functional capillary density with uniformly orthograde perfusion and faster red blood cell velocity and white blood cell velocity ($\Delta 50\%$), in comparison to untreated animals, as determined by spatio-temporal analysis (line-to-shift method) (Table 5).

Table 3. Functional Data of F344 Rat Kidneys Transplanted into LEW Rats, 10 Days after Transplantation

	Control	S247
Number (n)	9	8
Body weight (g)	224.4 $\pm$ 5.2	231.3 $\pm$ 7.7
Relative allograft weight (g/100 g/bw)	0.59 $\pm$ 0.013	0.57 $\pm$ 0.032
Relative endogenous kidney weight (g/100 g/bw)	0.47 $\pm$ 0.009	0.48 $\pm$ 0.019
Systolic arterial pressure (mmHg)	116 $\pm$ 1.3	116 $\pm$ 3.0
Proteinuria (mg/24 hours)	8.53 $\pm$ 0.98	4.12 $\pm$ 1.01*

Significant differences between untreated (control) and S247-treated allografts.

* $P < 0.05$.

Table 4. Functional Data of BN Rat Kidneys Transplanted into LEW Rats, 7 Days after Transplantation

	Control	S247
Number (n)	8	8
Body weight (g)	261.0 ± 4.5	251.8 ± 7.2
Relative allograft weight (g/100 g/bw)	0.49 ± 0.011	0.55 ± 0.041
Systolic arterial pressure (mmHg)	96.4 ± 3.1	96.5 ± 2.5
Serum creatinine (mg/dl)	4.26 ± 0.3	2.94 ± 0.4*
Proteinuria (mg/24 hours)	9.77 ± 0.78	6.61 ± 2.36*

Significant differences between untreated (control) and S247-treated allografts.

* $P < 0.05$.

Effects of S247 on Leukocyte-Endothelial Interactions in Vivo and in Vitro

Leukocyte emigration plays a central role in inflammatory and immune responses such as allograft rejection and is regulated by various chemotactic peptides (eg, chemokines) and adhesion molecules.^{10,38} Therefore the effects of α_v blockade on LEIs within the cortical peritubular capillary of renal allografts were analyzed. As shown in Figures 3 and 4, acute rejection of F344-LEW allografts in comparison with sham-operated F344 kidneys induced a significant increase of primary LEIs (rolling leukocytes), in firm cell adhesion to the vessel wall and in leukocyte transmigration across the endothelial border. S247 resulted in a significant decrease of all measured parameters (rolling $\Delta 50\%$, firm adherence $\Delta 65\%$, and transmigration $\Delta 60\%$) (Figure 4).

To corroborate the inhibitory action of α_v integrin antagonist on leukocyte-endothelial-interactions the effects of α_v integrin blockade were studied in an *in vitro* laminar flow system under strictly defined parameters. The interactions between HMEC-1 cells, unstimulated or stimulated with IL-1 β , and unstimulated THP-1 monocytes were evaluated under physiological flow conditions at a shear rate of 1.5 dyn/cm². S247 was added at two different concentrations: 0.1 and 0.5 μ mol/L. Endothelial cell activation by IL-1 β resulted in a significant increase of rolling and adherence of THP1 cells (Figure 5) compared with controls. The addition of S247 to prestimulated HMEC-1 cells revealed a significant and dose-dependent decrease of rolling ($\Delta 50\%$) as well as of shear resistant sticking of monocytic cells to the endothelium ($\Delta 60\%$).

Effects of S247 on Proliferation and Transmigration of Endothelial Cells, Macrophages, and Fibroblasts in Co-Culture with Kidney Tubular Cells

In rejecting kidney allografts, activated tubular cells are in close proximity to endothelial cells and monocytes in the peritubular capillaries and interstitial fibroblasts. Using co-culture models, we analyzed the paracrine effect of HK-2 tubular epithelia on proliferation of HDMECs and HDFs. Enhanced proliferation of both endothelial cells as well as fibroblasts was observed in the presence of HK-2 kidney cells. This paracrine promitotic stimulus was significantly inhibited by S247 (1 μ mol/L) ($P < 0.05$) (Figure 6A). The effects of S247 on HK-2-mediated transmigration of macrophages and fibroblasts were also investigated. We found that α_v integrin antagonism markedly reduced the transmigration of both cell types through Matrigel, taken as a model of extracellular matrix, by 50 to 80%; this effect was most prominent for the THP-1 cells ($P < 0.05$; Figure 6B).

Discussion

Acute rejection of renal allografts is characterized by endothelialitis/arteritis, tubulitis, and an interstitial mononuclear cell infiltrate³⁹; it is a model of acute inflammation with defined onset and composition of inflammatory cells, and its severity is dependent on major histocompatibility complex mismatch. This acute inflammatory process is a tightly regulated reaction characterized by activation, recruitment, and specific spatial distribution of inflammatory cells in response to alloantigens.¹⁰

Table 5. Effects of α_v Integrin Blockade by S247 on Microvascular Perfusion of Peritubular Capillaries within the Renal Cortex of F344 to Lewis Allografts 10 Days after Transplantation and Kidneys of Sham-Operated F344 Rats

	Sham	Control	S247
Capillary density (cm/cm ²)	384.1 ± 5.02	223.5 ± 14.49 ^{†††}	309.9 ± 14.73 ^{**}
RBCV (mm/second)	0.62 ± 0.03	0.31 ± 0.02 ^{†††}	0.49 ± 0.01 ^{***}
WBCV (mm/second)	0.61 ± 0.06	0.28 ± 0.02 ^{††}	0.41 ± 0.03 [*]

RBCV, red blood cell velocity; WBCV, white blood cell velocity.

* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ between untreated (control) and S247-treated allografts.

†† $P < 0.01$, and ††† $P < 0.001$ between sham-operated (sham) kidney and untreated (control) allografts.

Figure 3. A–F: Intravital microscopy. Microvascular perfusion of sham-operated and allogeneic transplanted F344 kidney 10 days after transplantation as shown by intravital microscopy. The kidney of a sham-operated rat shows well-preserved capillaries in the renal cortex (**A**); in an allogeneic kidney without treatment the functional capillary network is reduced (**B**, arrows); S247 treatment resulted in an increase of capillary perfusion (**C**). Leukocyte-endothelial-interaction (LEI) *in vivo*: almost no interaction between rhodamine-6-G-stained leukocytes and peritubular endothelium in a sham-operated kidney (**D**); in contrast, allogeneic transplanted kidneys showed enhanced LEI with an increase in firm adherence of leukocytes 10 days after transplantation (**E**, arrows). S247-treated allografts presented a strong reduction in the number of interactions between circulating leukocytes and the endothelium (**F**, arrows). Original magnifications: $\times 200$ (**A–C**); $\times 400$ (**D–F**).

α_v integrins have been shown to be involved in a variety of cell functions including cell migration, proliferation, and differentiation, which are central in the development of inflammation.^{3,6} With the exception of β_4 , integrins possess short cytoplasmic tails that bind directly or indirectly to a large number of cytoplasmic kinases, scaffolds, and cytoskeletal proteins (eg, focal adhesion kinase/FAK, Src family members) that support signals to and from integrins.^{40,41} $\alpha_v\beta_3$ and $\alpha_v\beta_5$ play important roles in regulating angiogenesis and cell migration; two other family members, $\alpha_v\beta_6$ and $\alpha_v\beta_8$, have specialized roles in activating TGF- β , suggesting that α_v integrins might be of importance in inflammation and fibrosis.⁴²

The five integrins that contain the α_v subunit are widely expressed, and their expression is tightly regulated. Increases in $\alpha_v\beta_3$ expression in acute cellular rejection of heart allografts and coronary graft vasculopathy in humans and in ischemic acute renal failure in rat have been observed.^{25–27} In ischemic rat kidneys β_1 and $\alpha_v\beta_3$ were found to be localized in proximal and distal tubules and endothelium.²⁷

Also different forms of glomerulonephritis were found to be associated with modifications in their α_v integrin expression: the distal tubular expression of β_3 was increased, and a *de novo* appearance of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ on proximal tubular cells in comparison to normal kidneys was noticed^{24,43}; $\alpha_v\beta_6$, which is exclusively expressed by epithelial cells, was elevated in the kidney epithelium of patients with membranous glomerulonephritis, IgA nephropathy, and in acute and chronic rejection of human kidney allografts.^{44,45} By immunostaining we could show

the increased expression of $\alpha_v\beta_3$ in rejecting rat kidney allografts, in peritubular capillaries, on the endothelial surface of preglomerular arteries and in tubular epithelial cells in comparison to normal F344 kidneys as well as its presence in the intra-graft perivascular cellular infiltrate. Ligands for α_v integrins, which include osteopontin, fibronectin, fibrinogen, and MMP-2, are also known to be expressed and increased in allograft rejection, partly by tubular epithelia and partly by interstitial cells.^{8,18,19} All these data led us to presume that α_v integrins might have potent inflammatory actions in acute renal allograft rejection, and abrogation of their functions might reduce rejection phenomena.

In two models of rat kidney transplantation, α_v integrin antagonism by the novel peptidomimetic α_v integrin antagonist S247 significantly ameliorated the histological signs of acute rejection and improved graft function. The mononuclear cell infiltrate, including ED1⁺ monocytes/macrophages, and CD8⁺ T lymphocytes was significantly decreased in comparison with untreated grafts.

Monocyte/macrophage accumulation within an acutely rejecting allograft occurs by recruitment and also by local proliferation. These two processes contribute to an amplification of the inflammatory response and destruction of the transplanted organ.⁴⁶ We have studied how the dynamics of these events are influenced by the blockade of α_v integrins *in vivo* and *in vitro*. S247 has been shown to exhibit inhibiting potency for $\alpha_v\beta_3$ (IC₅₀, 0.4 nmol/L) and at a twofold to fourfold higher IC₅₀ against other α_v integrins.³⁰ IC₅₀ values for other integrins ($\alpha_5\beta_1$, $\alpha_{11\beta}\beta_3$) are

Figure 4. A–C: Quantitative evaluation of LEIs of sham-operated kidneys of F344 rats and transplanted F344 to LEW kidneys without (control) and with S247 (S247) as determined by intravital microscopy: primary interaction of leukocytes with capillary endothelium (**A**), firm adherence of leukocytes to the capillary vessel wall (**B**), and transmigrated leukocytes into the tubulointerstitial space (**C**). In untreated allografts LEIs (rolling, sticking, and transmigration) were significantly increased 10 days after transplantation compared with sham-operated kidneys ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). In S247-treated renal allografts LEI was significantly decreased compared with untreated grafts ($^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). IVM, intravital microscopy; S247, α_v integrin antagonist.

much higher. Thus S247 is quite specifically inhibiting $\alpha_v\beta_3$.

Leukocyte recruitment is a multistep process that requires a series of leukocyte-endothelial adhesive interac-

Figure 5. A and B: *In vitro* laminar flow assay of monocytic THP1 cells on human microvascular endothelial cells (HMEC-1) in a parallel flow chamber at 1.5 dyn/cm². HMEC-1 cells were grown to confluence in Petri dishes and left either unstimulated (control) or stimulated with IL-1 β for 12 hours. Stimulation of endothelium with IL-1 β (10 ng/ml) for 12 hours resulted in a significant increase of rolling and firmly adherent THP1 cells (control, $^{***}P < 0.05$). Exposure of IL-1 β -activated endothelial cells to S247 for 60 minutes resulted in significant reduction of rolling and permanent adherence ($^{***}P < 0.05$). S247, α_v integrin antagonist.

tions: initial tethering of leukocytes to the vessel wall, followed by rolling along the endothelium, tight adhesion to the endothelial surface, and ultimately movement of the leukocyte through the intercellular junctions into the underlying tissue³⁸; this cascade is regulated by the sequential activation of adhesion molecules and their ligands.^{20,38} By intravital microscopy we could show that all these steps of LEIs were significantly decreased in S247-treated allografts in comparison to controls. Furthermore, monocyte-endothelial interaction events on IL-1 β -activated endothelial cells *in vitro* by a laminar flow assay were dose-dependently reduced.

The involvement of α_v integrins in transendothelial migration has been demonstrated. A β_3 integrin-deficient monocytic cell line displayed poor migratory ability compared with a β_3 integrin-positive monocytic cell line in a stationary transendothelial migration assay.²¹ Antibodies against α_v or integrin-associated protein (IAP, CD47), a 50-kd membrane protein found on a variety of different cell types, inhibited transmigration of β_3 -positive monocytes.^{21,47}

Figure 6. A and B: Proliferation and transmigration in co-culture transwell assays. **A:** Integrin antagonist S247 (1 μ mol/L) significantly inhibited the tubular epithelial cell (HK2)-induced transmigration of dermal fibroblasts (HDFs) and macrophages (THP1). Mean $\pm$ SD. * P < 0.05 versus control. **B:** Co-culture of tubular epithelial cells with HDMECs and HDFs enhanced proliferation of the latter two cell types. This HK2-mediated paracrine promotive effect was markedly inhibited by S247 treatment (1 μ mol/L). Mean $\pm$ SD. * P < 0.05, S247-treated versus nontreated HDMVC or HDF cells in co-culture.

Integrins do not work only as individual receptors but also as components of supramolecular complexes at the plasma membrane. These complexes can modulate surface expression levels as well as affinity states of the integrins with influence on their function and processes not achieved by the individual components of the complex alone.⁴¹ The IAP- $\alpha_v\beta_3$ complex appears to be particularly important in myeloid cell activation and migration across endothelial and epithelial monolayers; leukocyte PECAM-1 (CD31) has been shown to engage IAP- $\alpha_v\beta_3$ on the endothelium, leading to Ca^{2+} influx and to endothelial retraction with loss of the tight junction and leukocyte transmigration.⁴⁸ The positive effects of α_v integrin association with growth factor receptors such as platelet-derived growth factor receptor on cell migration has been reported.^{41,48} In addition, $\alpha_v\beta_3$ integrin has been shown

to influence cell migration and adhesion by modulating the activity/function of $\alpha_4\beta_1$ (VLA-4) as well as $\alpha_M\beta_2$ and $\alpha_L\beta_2$ on monocytes^{49,50}; these integrins are highly expressed in rejecting organs.⁵¹

Tubular cell activation in acute rejection results in the local expression of adhesion molecules (ICAM-1, osteopontin) and in the synthesis of cytokines/chemokines (eg, IL-15, TGF- β , RANTES).^{52,53} This in turn supports leukocyte infiltration and activation, fibroblast proliferation, and matrix synthesis.^{10,23} Because of the close proximity of tubular cells to microvascular endothelium and monocytes in peritubular capillaries, tubular epithelia will interact with these cells. Therefore we used co-culture models of HK-2 human tubular epithelial cells with primary human microvascular endothelial cells, fibroblasts, and macrophages. Tubular epithelial cells induced transmigration and proliferation in these cells, effects strongly inhibited by blocking α_v integrins by S247.

S247 has been shown to block $\alpha_v\beta_3$ -mediated cell migration and proliferation in different tumor models with high specificity.^{28,30} Occupancy of $\alpha_v\beta_3$ integrins can decrease monocyte binding capacity to ICAM-1 and VCAM-1, which are up-regulated in acute allograft rejection.^{21,49,51,54} We and others^{17,32} have shown that tubular osteopontin expression is significantly enhanced in renal biopsies with acute rejection and could be correlated with interstitial macrophage infiltration and cell proliferation. Osteopontin is an extracellular matrix protein containing an RGD sequence, which can interact with at least three different α_v -containing integrins, $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$; its migratory activity seems to depend on the presence of a functional $\alpha_v\beta_3$ on the cell surface.^{55,56} Recently, it has also been shown that osteopontin-mediated alveolar epithelial cell migration and fibroblast migration and proliferation could be inhibited by an antibody to $\alpha_v\beta_3$ integrin.⁵⁷ These data confirm the results with a decrease in tubular epithelia-induced migration of macrophages and fibroblasts by S247.

A characteristic feature of interstitial rejection is tubulitis, in which the inflammatory cell crosses the tubular basement membrane and comes into close contact with the tubular epithelial cell contributing to tubular cell activation.⁵² Acute kidney allograft rejection leads to alterations in the expression and activity of different MMPs, such as the basement membrane-degrading MMP-2, a known ligand for $\alpha_v\beta_3$.¹⁹ The multiprotein MMP-activating complex also includes integrin $\alpha_v\beta_3$.⁵⁸ Negative regulation of integrin/protease binding, as should be the case by S247 treatment, can prevent excessive degradation of ECM and might inhibit cell migration through the tubular basement membrane.⁵⁹ Reduced cell proliferation was observed also *in vivo*: the number of intragraft Ki-67-positive cells decreased significantly in S247 grafts; the anti-proliferative effect was more evident in interstitial cells. This finding could reflect the reduced number of infiltrating cells but was most probably attributable to the direct effect of α_v integrin inhibition.

Numerous vimentin⁺ and α -SMA⁺ fibroblasts could be seen in the interstitium of kidney allografts in both major histocompatibility complex disparate transplantation models; they were reduced by S247. The degree of myo-

fibroblast proliferation early in the period after transplantation has been found to be predictive for late chronic changes, providing a link between acute rejection and development of chronic allograft nephropathy.⁶⁰ These observations might predict a possible benefit of α_v integrin blockade in preventing chronic rejection.

The prominent effect of S247 on the number of CD8⁺ cells in treated allografts suggest that these cells are relevant targets of anti- α_v integrin therapy. In the early immunological phase of the alloresponse, T-cell activation is a crucial event. Previous experiments have emphasized the importance of some integrins in the activation of lymphocytes. $\alpha_v\beta_3$ has been shown to be a T-cell activation antigen in the mouse.^{61,62} Furthermore, $\alpha_v\beta_3$ has been demonstrated to provide the primary signal in the T-cell activation process for IL-2 release in the absence of antigen recognition by the T-cell receptor complex.⁶³ The same study has shown that IL-2 secretion could be blocked by an antibody directed against $\alpha_v\beta_3$. The exact role of α_v integrin-dependent signaling in the activation of lymphocytes in allograft rejection still has to be clarified.

In the BN-L model of kidney allograft rejection, the addition of S247 to low-dose CsA decreased rejection signs, the number of infiltrating CD8⁺ T lymphocytes, and significantly improved graft function compared with CsA therapy alone ($P < 0.05$). Given that CsA-induced nephrotoxicity is a major clinical problem, a reduced CsA dose in combination with α_v integrin blockade could be of benefit.

In summary, we demonstrated the importance of α_v integrins in acute renal allograft rejection. The characteristic cellular responses of acute rejection that were blocked by the inhibition of α_v -containing integrins included adhesion, migration, and proliferation of mononuclear inflammatory cells and resident interstitial cells. Thereby, integrin α_v blockade might be used in anti-rejection therapy.

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References

- Noti JD: Expression and function of the leukocyte integrins in organ transplant rejection. *Curr Med Chem* 2004, 11:477–490
- Dedrick RL, Bodary S, Garovoy MR: Adhesion molecules as therapeutic targets for autoimmune diseases and transplant rejection. *Expert Opin Biol Ther* 2003, 3:85–95
- Hynes RO: Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 1992, 69:11–25
- Berton G, Lowell CA: Integrin signalling in neutrophils and macrophages. *Cell Signal* 1999, 11:621–635
- Bader BL, Rayburn H, Crowley D, Hynes RO: Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all α_v integrins. *Cell* 1998, 13:507–519
- Eliceiri BP, Cheresh DA: The role of α_v integrins during angiogenesis: insights into potential mechanisms of action and clinical development. *J Clin Invest* 1999, 103:1227–1230
- Tucker GC: Inhibitors of integrins. *Curr Opin Pharmacol* 2002, 2:394–402
- Byzova TV, Rabbani R, D'Souza SE, Plow EF: Role of integrin $\alpha_v\beta_3$ in vascular biology. *Thromb Haemost* 1998, 80:726–734
- Cheresh DA: Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. *Proc Natl Acad Sci USA* 1987, 84:6471–6475
- Le Moine A, Goldman M, Abramowicz D: Multiple pathways to allograft rejection. *Transplantation* 2002, 73:1373–1381
- Karamessinis PM, Tzinia AK, Kitsiou PV, Stettler-Stevenson WG, Michael AF, Fan WW, Zhou B, Margaritis LH, Tsilibary EC: Proximal tubular epithelial cell integrins respond to high glucose by altered cell-matrix interactions and differentially regulate matrixin expression. *Lab Invest* 2002, 82:1081–1093
- Kitazawa S, Ross FP, McHugh K, Teitelbaum SL: Interleukin-4 induces expression of the integrin $\alpha_v\beta_3$ via transactivation of the β_3 gene. *J Biol Chem* 1995, 270:4115–4120
- Hoffmann S, He S, Jin M, Ehren M, Wiedemann P, Ryan SJ, Hinton DR: A selective cyclic integrin antagonist blocks the integrin receptors $\alpha_v\beta_3$ and $\alpha_v\beta_5$ and inhibits retinal pigment epithelium cell attachment, migration and invasion. *BMC Ophthalmol* 2005, 5:16
- Hermann P, Armant M, Brown E, Rubio M, Ishihara H, Ulrich D, Caspary RG, Lindberg FP, Armitage R, Maliszewski C, Delespesse G, Sarfati M: The vitronectin receptor and its associated CD47 molecule mediates proinflammatory cytokine synthesis in human monocytes by interaction with soluble CD23. *J Cell Biol* 1999, 144:767–775
- Hahn AB, Kasten-Jolly JC, Constantino DM, Graffunder E, Singh TP, Shen GK, Conti DJ: TNF- α , IL-6, IFN- γ , and IL-10 gene expression polymorphisms and the IL-4 receptor α -chain variant Q576R: effects on renal allograft outcome. *Transplantation* 2001, 72:660–665
- Reinders ME, Sho M, Izawa A, Wang P, Mukhopadhyay D, Koss KE, Geehan CS, Luster AD, Sayegh MH, Briscoe DM: Proinflammatory functions of vascular endothelial growth factor in alloimmunity. *J Clin Invest* 2003, 112:1655–1665
- Alchi B, Nishi S, Kondo D, Kaneko Y, Matsuki A, Imai N, Ueno M, Iguchi S, Sakatsume M, Narita I, Yamamoto T, Gejyo F: Osteopontin expression in acute renal allograft rejection. *Kidney Int* 2005, 67:886–896
- Paczek L, Bartłomiejczyk I, Gradowska L, Lao M, Gorski A, Morzycka-Michalik M, Gaciong Z: Increased content of fibronectin and laminin in glomeruli isolated from chronically rejected human renal allografts. *Transplantation* 1996, 61:654–655
- Ermolli M, Schumacher M, Lods N, Hammoud M, Marti HP: Differential expression of MMP-2/MMP-9 and potential benefit of an MMP inhibitor in experimental acute kidney allograft rejection. *Transpl Immunol* 2003, 11:137–145
- Springer TA: Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994, 76:301–314
- Weerasinghe D, McHugh KP, Ross FP, Brown EJ, Gisler RH, Imhof BA: A role for the $\alpha_v\beta_3$ integrin in the transmigration of monocytes. *J Cell Biol* 1998, 142:595–607
- Thompson RD, Wakelin MW, Larbi KY, Dewar A, Asimakopoulos G, Horton MA, Nakada MT, Nourshargh S: Divergent effects of platelet-endothelial cell adhesion molecule-1 and β_3 integrin blockade on leukocyte transmigration in vivo. *J Immunol* 2000, 165:426–434
- Waller JR, Nicholson ML: Molecular mechanisms of renal allograft fibrosis. *Br J Surg* 2001, 88:1429–1441
- Roy-Chaudhury P, Hillis G, McDonald S, Simpson JG, Power DA: Importance of the tubulointerstitium in human glomerulonephritis. II. Distribution of integrin chains β_1 , α_1 to 6 and α_v . *Kidney Int* 1997, 52:103–110
- Yamani MH, Yang J, Masri CS, Ratliff NB, Bond M, Starling RC, McCarthy P, Plow E, Young JB: Acute cellular rejection following human heart transplantation is associated with increased expression of vitronectin receptor (integrin $\alpha_v\beta_3$). *Am J Transplant* 2002, 2:129–133
- Yamani MH, Masri CS, Ratliff NB, Bond M, Starling RC, Tuzcu EM, McCarthy PM, Young JB: The role of vitronectin receptor ($\alpha_v\beta_3$) and tissue factor in the pathogenesis of transplant coronary vasculopathy. *J Am Coll Cardiol* 2002, 39:804–810
- Romanov V, Noiri E, Czerwinski G, Finsinger D, Kessler H, Goligorsky MS: Two novel probes reveal tubular and vascular Arg-Gly-Asp

- (RGD) binding sites in the ischemic rat kidney. *Kidney Int* 1997, 52:93–102
28. Abdollahi A, Griggs DW, Zieher H, Roth A, Lipson KE, Saffrich R, Grone HJ, Hallahan DE, Reisfeld RA, Debus J, Niethammer AG, Huber PE: Inhibition of $\alpha_v\beta_3$ integrin survival signaling enhances antiangiogenic and antitumor effects of radiotherapy. *Clin Cancer Res* 2005, 11:6270–6279
 29. Abdollahi A, Li M, Ping G, Plathow C, Domhan S, Kiessling F, Lee LB, McMahon G, Grone HJ, Lipson KE, Huber PE: Inhibition of platelet-derived growth factor signaling attenuates pulmonary fibrosis. *J Exp Med* 2005, 201:925–935
 30. Shannon KE, Keene JL, Settle SL, Duffin TD, Nickols MA, Westlin M, Schroeter S, Ruminski PG, Griggs DW: Anti-metastatic properties of RGD-peptidomimetic agents S137 and S247. *Clin Exp Metastasis* 2004, 21:129–138
 31. Lee S: An improved technique of renal transplantation in the rat. *Surgery* 1967, 61:771–773
 32. Bedke J, Kiss E, Schaefer L, Behnes CL, Bonrouhi M, Gretz N, Horuk R, Diedrichs-Moehring M, Wildner G, Nelson PJ, Grone HJ: Beneficial effects of CCR1 blockade on the progression of chronic renal allograft damage. *Am J Transplant* 2007, 7:527–537
 33. Gröne HJ, Helmchen U: Impairment and recovery of the clipped kidney in two kidney, one clip hypertensive rats during and after antihypertensive therapy. *Lab Invest* 1986, 54:645–655
 34. Kiss E, Adams J, Grone HJ, Wagner J: Isotretinoin ameliorates renal damage in experimental acute renal allograft rejection. *Transplantation* 2003, 76:480–489
 35. Adams J, Kiss E, Arroyo AB, Bonrouhi M, Sun Q, Li Z, Gretz N, Schnitger A, Zouboulis CC, Wiesel M, Wagner J, Nelson PJ, Grone HJ: 13-cis Retinoic acid inhibits development and progression of chronic allograft nephropathy. *Am J Pathol* 2005, 167:285–298
 36. Klyscz T, Junger M, Jung F, Zeintl H: Cap image—a new kind of computer-assisted video image analysis system for dynamic capillary microscopy. *Biomed Tech (Berl)* 1997, 42:168–175
 37. Gröne HJ, Weber C, Weber KS, Grone EF, Rabelink T, Klier CM, Wells TN, Proudfoot AE, Schlondorff D, Nelson PJ: Met-RANTES reduces vascular and tubular damage during acute renal transplant rejection: blocking monocyte arrest and recruitment. *FASEB J* 1999, 13:1371–1383
 38. Kriegelstein CF, Granger DN: Adhesion molecules and their role in vascular disease. *Am J Hypertens* 2001, 14:44S–54S
 39. Solez K, Axelsen RA, Benediktsson H, Burdick JF, Cohen AH, Colvin RB, Croker BP, Droz D, Dunnill MS, Halloran PF, Häyry P, Jennette JC, Keown PA, Marcussen N, Mihatsch MJ, Morozumi K, Myers BD, Nast CC, Olsen S, Racusen LC, Ramos EL, Rosen S, Sachs DH, Salomon DR, Sanfilippo F, Verani R, von Willebrand E, Yamaguchi Y: International standardization of criteria for the histologic diagnosis of renal allograft rejection: the Banff working classification of kidney transplant pathology. *Kidney Int* 1993, 44:411–422
 40. Liu S, Calderwood DA, Ginsberg MH: Integrin cytoplasmic domain-binding proteins. *J Cell Sci* 2000, 113:3563–3571
 41. van der Flier A, Sonnenberg A: Function and interactions of integrins. *Cell Tissue Res* 2001, 305:285–298
 42. Sheppard D: Roles of α_v integrins in vascular biology and pulmonary pathology. *Curr Opin Cell Biol* 2004, 16:552–557
 43. Hafdi Z, Lesavre P, Nejari M, Halbwachs-Mecarelli L, Droz D, Noël LH: Distribution of $\alpha_v\beta_3$, $\alpha_v\beta_5$ integrins and the integrin associated protein-IAP (CD47) in human glomerular diseases. *Cell Adhes Commun* 2000, 7:441–451
 44. Hahn K, Lukashev ME, Luo Y, Yang WJ, Dolinski BM, Weinreb PH, Simon KJ, Chun Wang L, Leone DR, Lobb RR, McCrann DJ, Allaire NE, Horan GS, Fogo A, Kalluri R, Shield CF, Sheppard D, Gardner HA, Violette SM: $\alpha_v\beta_6$ integrin regulates renal fibrosis and inflammation in Alport mouse. *Am J Pathol* 2007, 170:110–125
 45. Trevillian P, Paul H, Millar E, Hibberd A, Agrez MV: $\alpha_v\beta_6$ integrin expression in diseased and transplanted kidneys. *Kidney Int* 2004, 66:1423–1433
 46. Jose MD, Ikezumi Y, van Rooijen N, Atkins RC, Chadban SJ: Macrophages act as effectors of tissue damage in acute renal allograft rejection. *Transplantation* 2003, 76:1015–1022
 47. Lindberg FP, Gresham HD, Reinhold MI, Brown EJ: Integrin-associated protein immunoglobulin domain is necessary for efficient vitronectin bead binding. *J Cell Biol* 1996, 134:1313–1322
 48. Porter JC, Hogg N: Integrins take partners: cross-talk between integrins and other membrane receptors. *Trends Cell Biol* 1998, 8:390–396
 49. Imhof BA, Weerasinghe D, Brown EJ, Lindberg FP, Hammel P, Piali L, Dessing M, Gisler R: Cross talk between $\alpha_v\beta_3$ and $\alpha_4\beta_1$ integrins regulates lymphocyte migration on vascular cell adhesion molecule. *Eur J Immunol* 1997, 27:3242–3252
 50. Capo C, Lindberg FP, Meconi S, Zaffran Y, Tardei G, Brown EJ, Raoult D, Mege JL: Subversion of monocyte functions by *Coxiella burnetii*: impairment of the cross-talk between $\alpha_v\beta_3$ integrin and CR3. *J Immunol* 1999, 163:6078–6085
 51. Kloover JS, Soots AP, Krogerus LA, Kauppinen HO, Loginov RJ, Holma KL, Bruggeman CA, Ahonen PJ, Lautenschlager IT: Rat cytomegalovirus infection in kidney allograft recipients is associated with increased expression of intracellular adhesion molecule-1, vascular adhesion molecule-1, and their ligands leukocyte function antigen-1 and very late antigen-4 in the graft. *Transplantation* 2000, 69:2641–2647
 52. Robertson H, Kirby JA: Post-transplant renal tubulitis: the recruitment, differentiation and persistence of intra-epithelial T cells. *Am J Transplant* 2003, 3:3–10
 53. Baer PC, Scherberich JE, Bereiter-Hahn J, Geiger H: Induction of RANTES, HLA-DR, and intercellular adhesion molecule-1 on highly purified distal tubular cells from human kidney. *Transplantation* 2000, 69:2456–2459
 54. Stojanovic T, Grone HJ, Gieseler RK, Klanke B, Schlemminger R, Tsikas D, Grone EF: Enhanced renal allograft rejection by inhibitors of nitric oxide synthase: a nonimmunologic influence on alloreactivity. *Lab Invest* 1996, 74:496–512
 55. Liaw L, Skinner MP, Raines EW, Ross R, Cheresch DA, Schwartz SM, Giachelli CM: The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of $\alpha_v\beta_3$ in smooth muscle cell migration to osteopontin in vitro. *J Clin Invest* 1995, 95:713–724
 56. Denhardt DT, Noda M, O'Regan AW, Pavlin D, Berman JS: Osteopontin as a means to cope with environmental insults: regulation of inflammation, tissue remodeling, and cell survival. *J Clin Invest* 2001, 107:1055–1061
 57. Pardo A, Gibson K, Cisneros J, Richards TJ, Yang Y, Becerril C, Yousem S, Herrera I, Ruiz V, Selman M, Kaminski N: Up-regulation and profibrotic role of osteopontin in human idiopathic pulmonary fibrosis. *PLoS Med* 2005, 2:e251
 58. Brooks PC, Stromblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, Quigley JP, Cheresch DA: Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin $\alpha_v\beta_3$. *Cell* 1996, 85:683–693
 59. Silletti S, Kessler T, Goldberg J, Boger DL, Cheresch DA: Disruption of matrix metalloproteinase 2 binding to integrin $\alpha_v\beta_3$ by an organic molecule inhibits angiogenesis and tumor growth in vivo. *Proc Natl Acad Sci USA* 2001, 98:119–124
 60. Pilmore HL, Painter DM, Bishop GA, McCaughan GW, Eris JM: Early up-regulation of macrophages and myofibroblasts: a new marker for development of chronic renal allograft rejection. *Transplantation* 2000, 69:2658–2662
 61. Moulder K, Roberts K, Shevach EM, Coligan JE: The mouse vitronectin receptor is a T cell activation antigen. *J Exp Med* 1991, 173:343–347
 62. Roberts K, Yokoyama WM, Kehn PJ, Shevach EM: The vitronectin receptor serves as an accessory molecule for the activation of a subset of $\gamma\delta$ T cells. *J Exp Med* 1991, 173:231–240
 63. Stürmhöfel K, Brando C, Martinon F, Shevach EM, Coligan JE: Antigen-independent, integrin-mediated T cell activation. *J Immunol* 1995, 154:2104–2111