Originals

Małgorzata Wągrowska-Danilewicz, Marian Danilewicz

Immunohistochemical Study of Endothelin-1 (ET-1) in Human Acute Renal Allograft Rejection*

Department of Nephropathology, Chair of Pathomorphology, Medical University, Łódź

Immunoperoxidase staining was carried out using monoclonal antibody against endothelin-1 on renal allograft biopsy specimens from thirty one patients with acute renal transplant rejection: Banff 97 score IA (n=11), score IB (n=10) and score IIA (n=10). As a control 10 biopsy specimens of the kidneys removed because of trauma were used. Endothelin-1 immunostaining was investigated in the renal interstitial vasculature and tubular epithelium. In the normal kidney the immunoreactivity of endothelin-1 was demonstrated in vascular endothelial cells and tubular epithelial cells. The intensity of endothelin-1 staining in endothelial cells in renal allograft biopsies from patients with Banff score IA and IB was similar to controls. In biopsies with Banff score IIA a marked decrease in endothelin-1 immunostaining of endothelial cells was seen. Moreover, renal tubular epithelium from patients with acute allograft rejection with Banff score IB and IIA had elevated endothelin-1 immunoreactivity as compared with controls. The results showed altered endothelin-1 immunostaining in renal biopsy specimens in patients with acute transplant rejection. In acute tubulointerstitial rejection Banff IB and acute vascular rejection Banff IIA up-regulation of endothelin-1 on tubular epithelial cells was present, meanwhile in acute vascular rejection diminished immunoreactivity of endothelin-1 on endothelial cells was noted. In conclusion, our study suggests that endothelin-1 play an important role in renal tissue injury in acute tubulointerstitial and vascular allograft rejection.

Introduction

Acute allograft renal rejection (AAR) may occur within days of transplantation, or may appear suddenly months or even years later after immunosupression has been employed. The elevation of serum creatinine level followed by clinical signs of renal failure may point to acute allograft rejection. Histological evaluation of biopsy specimens is considered one of the most valuable tools in monitoring the transplant. *Tubulitis* and *vasculitis* are the cardinal features of acute renal graft rejection. Banff classification [18] for renal allo-graft rejection graded *tubulitis* and *vasculitis*, however several studies have concluded that the presence of vasculitis in a renal allograft biopsy is associated with poorer response to therapy and outcome. In spite of progress in understanding of pathogenetic mechanisms of renal injury during transplantation, the role of endothelin in this condition remain poorly understood. Endothelin (ET) is a potent biological mediator which exists in three isoforms: endothelin-1, -2 and -3, however ET-1 is considered the clinically most important endothelin in human kidney disease [11, 25]. In addition to endothelial cells, a variety of cell types in the kidney are able to produce ET-1 [7, 15]. Endothelins belong to a family of 21 amino acid peptides with two disulphide bridges [11], and exert their biologic effect *via* activation of specific membrane-bound receptors that consist of seven transmembrane domains [12]. Two different receptor subtypes have been cloned in mammalian tissues, termed ET(A) and ET(B) receptors [20]. The main vascular effects of endothelin are transient vasodilatation and profound and sustained vasoconstriction [8]. Systemic infusion of endothelin-1 (ET-1) in humans *in vivo* leads to blood pressure increase, sodium retention and reduction in urine flow [17, 23]. The renal vasculature is up to 10 times more sensitive to the vasoconstriction effects of endothelin-1 as compared with other vascular beds [2]. Several experimental studies suggest a pathophysiologic role of endothelin in renal failure and in the pathogenesis of renal allograft rejection [3, 13, 14, 19, 22, 24]. In kidney allografts endothelial impairment does occur in acute and chronic rejection, acute tubular necrosis and cyclosporine toxicity.

The aim of the present study was to compare the immunoreactivity of ET-1 in endothelium of peritubular capillaries and arterioles and epithelial tubular cells in renal allograft biopsies from patients with varying intensity of acute tubulointerstitial and vascular rejection graded according to Banff 97 classification.

Material and Methods

Patients

Thirty one renal allograft biopsy specimens from patients with AAR were examined by percutaneous renal biopsy. All biopsies had been performed solely for diagnostic purposes. All of our patients were adults: the mean age was 42.3±9.8 and male to female ratio was 18:13. The specimens were taken from 7 days to 5 months after engraftment (mean 49.5 days). Acute rejection was considered clinically when patients showed unexplained increase in serum creatinine concentration. Morphological diagnosis of AAR was established independently by two experienced nephropathologists according to Banff criteria [18] and based on light microscopy and immunofluorescence using standard protocols. Eleven specimens had Banff IA score: cases with interstitial infiltration (>25% of parenchyma affected) and foci of moderate *tubulitis* (>4 mononuclear cells/tubular cross section or group of 10 tubular cells). Ten specimens had IB score: cases with interstitial infiltration (>25% of parenchyma affected) and foci of severe *tubulitis* (>10 mononuclear cells/tubular cross section or group of 10 tubular cells). Ten specimens were classified as IIA score: cases with mild to moderate intimal arteritis in at least one arterial cross section. In all cases the standard immunosupression protocol was used. As a control 10 biopsy specimens of the kidneys removed because of trauma were used (the male to female ratio was 7:3, the mean age was 38.1±7.2). None of the persons from whom renal tissue originated was known to have previous or actual renal disease. All control specimens were histologically examined by two nephropathologists and found to be normal renal tissue.

Immunohistochemistry

Paraffin sections were mounted onto superfrost slides, deparaffinized and antigen retrieval Streptavidin-biotin complex (StreptABC) technique was employed. After rehydratation sections were incubated for 5 minutes with 3% hydrogen peroxide in distilled water, rinsed in Tris-buffered saline (TBS) and after blocking by normal rabbit serum (DAKO, Glostrup, Denmark) for 20min (dilution 1:5), the sections were incubated with monoclonal mouse anti-human anti-endothelin-1 antibody (clone TR .ET.48.5, Sigma, Saint Louis, USA, dilution 1:250) in a moist chamber for 1 hour at room temperature. Afterwards, sections were rinsed in TBS and incubated with biotinylated rabbit anti-mouse immunoglobulin (DAKO, Glostrup, Denmark) diluted 1:600 in TBS for 20min and StreptABComplex/HRP (DAKO, Glostrup, Denmark) for 30min prepared according to the instructions of the manufacturer. Visualisation was performed by incubating the sections in a solution of 0.5mg 3,3'-diaminobenzidine (DAKO, Glostrup, Denmark) per 1ml Tris-HCl buffer, pH 7.6, containing 0.02% hydrogen peroxide, for 10min. After washing, sections were counterstained with hematoxylin and coversliped. Negative controls were carried out by incubation in the absence of the primary antibody and always yielded negative results.

In each specimen staining intensity of ET-1 in the endothelium of peritubular capillaries and arterioles and in the renal tubular epithelium cells were recorded by two independent observers in 7 - 10 adjacent high power fields and graded as 0 (staining not detectable), 1 (minimal immunostaining in some cells), 2 (weak immunostaining intensity in all cells) and 3 (strong staining in all cells). The mean grade was calculated by averaging grades assigned by the two authors and approximating the arithmetical mean to the nearest unity.

Statistical methods

All data are expressed as mean±SD (standard deviation). Differences between groups were tested using unpaired Student's test preceded by evaluation of normality and Levene's test. The Mann-Whitney U test was used where appropriate. Results were considered statistically significant if $P<0.05$.

Results

The mean values of immunoexpression of ET-1 are shown in Table 1.

ET-1 immunostaining in normal controls: In endothelium of peritubular capillaries and renal arterioles a strong immunoreactivity of ET-1 was seen. In epithelium of renal tubules slight to moderate immunostaining of ET-1 was detected (Fig. 1).

ET-1 immunostaining in AAR Banff IA: The mean staining intensity in endothelial cells of peritubular capil-

TABLE 1

Endothelin-1-immunostaining in renal endothelium and tubular epithelial cells in controls and renal allograft biopsies with acute rejection (AAR). Data are expressed as mean±SD

	Capillaries	Arterioles	Tubules
Controls (n=10)	2.7±0.4	2.1±0.2	1.7±0.3
AAR Banff IA (n=11)	2.4±0.3	1.9±0.3	1.9±0.2
AAR Banff IB (n=10)	2.5±0.4	1.9±0.4	2.4±0.3*** (P<0.001)
AAR Banff IIA (n=10)	1.2±0.2* (P<0.001)	0.7±0.3** (P<0.001)	2.3±0.2**** (P<0.001)

   *AAR Banff IIA vs. all other groups and controls
  **AAR Banff IIA vs. all other groups and controls
 ***AAR Banff IB vs. AAR Banff IA and controls
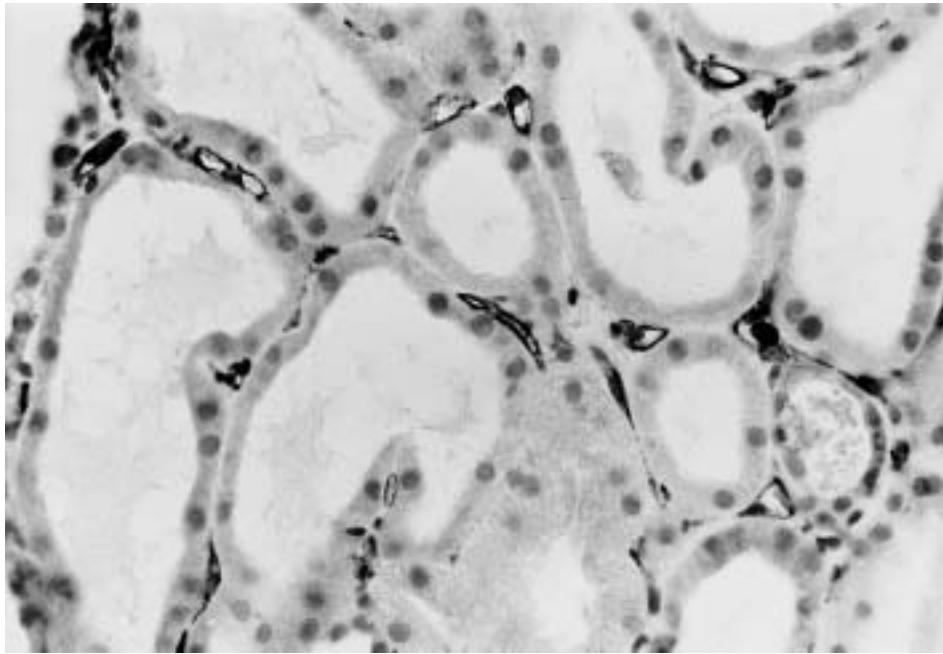****AAR Banff IIA vs. AAR Banff IA and controls

96

Fig. 1. Endothelin-1 immunostaining pattern in control kidney. Strong staining of endothelium in peritubular capillaries and slight immunostaining of epithelial tubular cells. Magn. 400x.
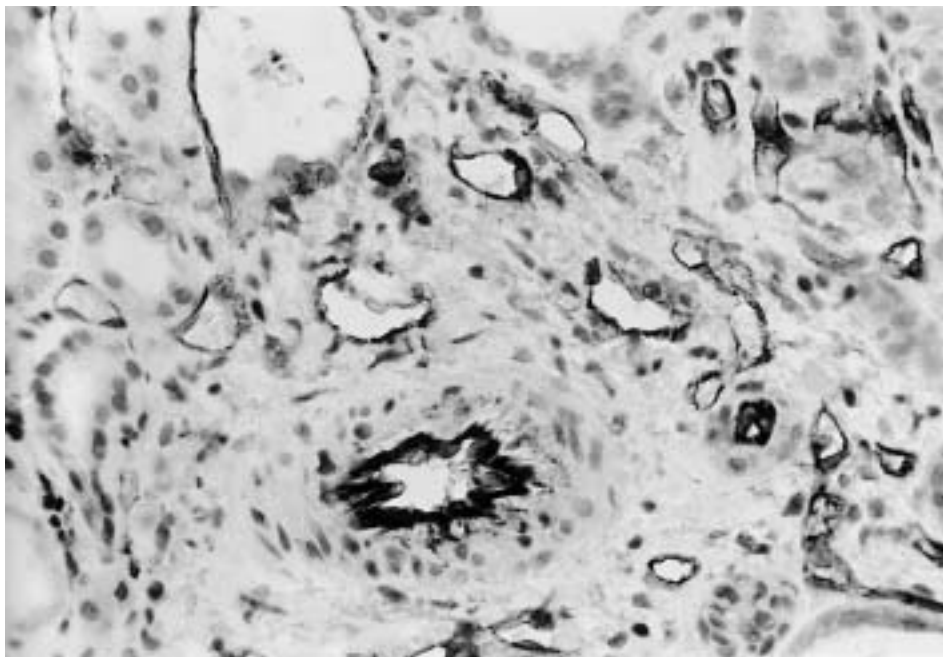

Fig. 2. Strong endothelin-1 immunostaining in endothelium of peritubular capillaries and artery in renal biopsy specimen in acute tubulointerstitial allograft rejection Banff IA. Magn. 400x.

laries and arterioles, and mean immunoreactivity of epithelial tubular cells were similar to staining intensity seen in normal controls (Fig. 2).

ET-1 immunostaining in AAR Banff IB: The mean staining immunoreactivity in endothelium of capillaries and arterioles did not differ significantly from normal kidneys and from renal tissue in AAR Banff IA. However, tubular staining was more prominent (Fig. 3) as compared with control renal tissue ($P<0.001$), and kidney specimens from patients with AAR Banff IA ($P<0.001$).

ET-1 immunostaining in AAR Banff IIA: The mean values of the endothelial immunostaining in capillaries and arterioles were significantly decreased (Fig. 4) in compari-

son with controls and renal tissue with AAR Banff IA and IB ($P<0.001$). Mean immunoreactivity of epithelial tubular cells were elevated as compared with control kidneys ($P<0.001$), and renal biopsy specimens in AAR Banff IA ($P<0.001$), however similar to staining intensity seen in AAR Banff IB ($P=0.39$, NS).

Discussion

Our study revealed the alterations in immunoreactivity of ET-1 in endothelium of peritubular capillaries and arterioles and epithelium of renal tubuli in AAR in transplanted kidneys. In vascular acute rejection Banff IIA there was a
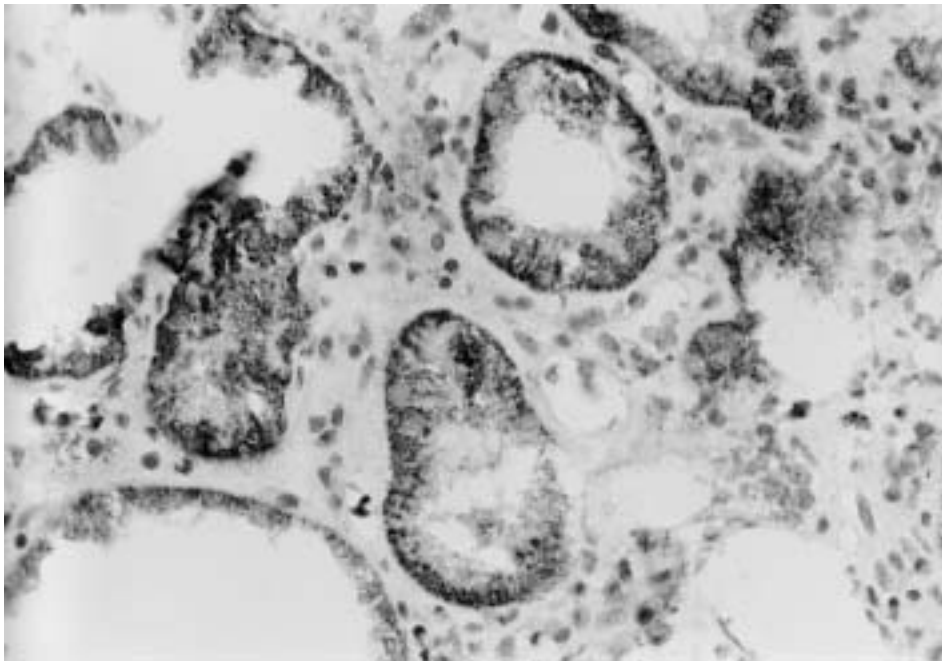
Fig. 3. Elevated ET-1 immuno-staining in renal tubular epithelium in acute tubulointerstitial allograft rejection Banff IB. Magn. 400x.
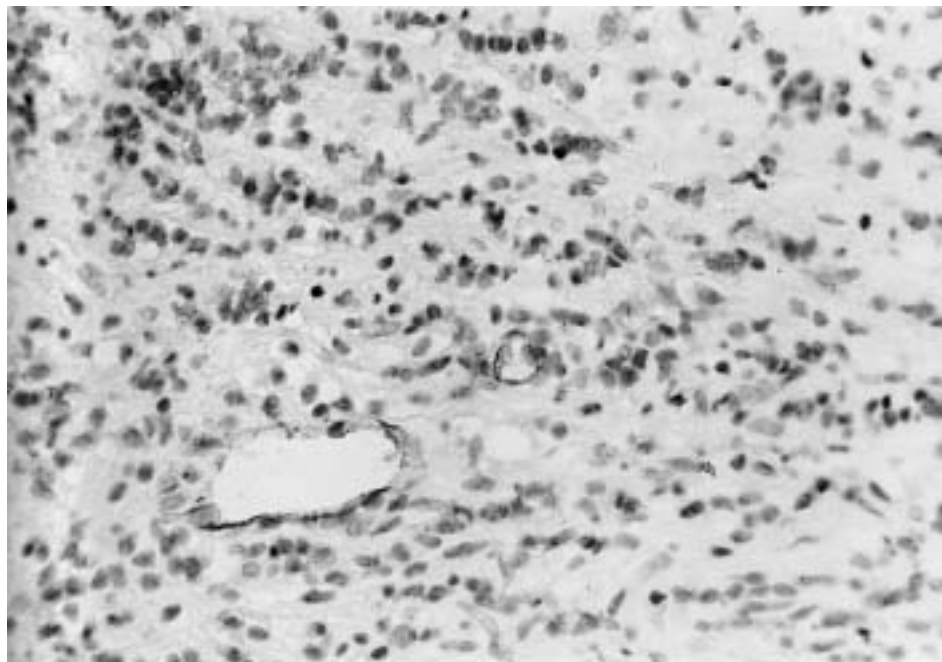

Fig. 4. Decreased ET-1 immuno-staining in endothelium of peritu-bular capillaries in renal biopsy specimen in acute vascular rejec-tion Banff IIA. Magn. 400x.

significant decrease in ET-1 staining intensity in endothe-lial cells. In cases of severe acute tubulointerstitial rejec-tion referred to IB Banff classification and in cases with mild to moderate vascular acute rejection Banff IIA there was increased ET-1 staining intensity in tubular epithe-lium. Endothelial and epithelial ET-1 immunostaining was not altered in cases with mild tubulointerstitial acute rejection Banff IA. Our results pointed to decreased en-dothelin-1 immunoreactivity in endothelial cells in vascu-lar acute allograft rejection and are in concordance with others observations. Watschinger et al. [24] revealed a loss of ET immunoreactivity in intrarenal vascular endo-thelium in 80% of biopsies showing signs of vascular

rejection, and what is more during rejection episodes plasma endothelin-1 level was elevated. These authors showed that significant reduction of ET-immunostaining in tubu-lar epithelial cells occurred in vascular rejection, but not in interstitial rejection. In contrast, in our study elevated ET-1 immunostaining in tubular epithelium was seen in vascular rejection and in severe interstitial rejection, but in biopsies with mild interstitial rejection ET-1 staining intensity in tubular epithelial cells did not differ from control kidneys. Chareandee et al. [1] reported similar results concerning epithelial ET-1 immunoreactivity in acute allograft rejection. These authors observed elevated tubular ET-1 staining in all 18 study patients with acute

rejection and in 5 out of 7 patients with chronic rejection. Unfortunately, their observations referred to acute allograft rejection without distinguishing between interstitial or vascular rejection. It is also worthy of note that in all previous ET-1 immunostaining study in renal biopsies from patients with renal allograft rejection the authors did not referred intensity of ET-1 immunoreactivity in respect to Banff classification. Thus, it may be the reason for the divergent results. It is obvious, that the intensity of renal tissue damage varies between IA and IB Banff allograft rejection score. In Banff IA only mild tubulointerstitial changes are seen, while in Banff IB severe tubulointerstitial damage is noted. In our study cases with vascular acute rejection referred to Banff IIA (mild to moderate intimal arteritis); but we did not evaluated ET-1 immunoreactivity in cases with severe arteritis or cases with transmural arteritis and arterial fibrinoid change or necrosis of medial smooth muscle cells. What is more, the differences in tissue processing and antibodies used may also reflect differences between our results and others. It is well known that endothelin is an important mediator of pathophysiologic alterations in renal hemodynamics. ET actions in the kidney are mediated primarily by paracrine and autocrine mechanisms [9]. In normal human kidney renal vessels are an important source of intrarenal ET-1 secretion [5], however, several studies documented ET-1 synthesis by renal tubular epithelium [5, 21]. Experimental studies in animals showed that ET-1 helps control the reabsorption of sodium in proximal tubules and water in cortical collecting ducts [5, 21]. Tubular ET-1 has also been proposed to mediate tubulointerstitial injury by regulating cell proliferation, matrix accumulation, and regional blood flow [4, 16]. Release of ET-1 from endothelial cells is stimulated by many vasoactive agents such as bradykinin, angiotensin II, vasopressin and inflammatory agents - interleukin-1, tumor growth factor-beta, tumor necrosis factor, thrombin [6, 10, 25, 26]. It is obvious, that renal vasculature and renal tubules are important site of injury in acute allograft rejection of transplanted kidneys. Endothelium constitutes one of the main targets for the infiltrating leukocytes, moreover severe pathological changes including swelling, necrosis, thrombosis, platelet and fibrin aggregation are indicative of direct endothelial damage. On the other hand, lymphocytic infiltration of tubular epithelium (*tubulitis*) and tubular damage are demonstrated in all patients with acute interstitial rejection.

In summary, our results revealed altered ET-1 immunoreactivity in endothelial and tubular epithelial cells in Banff IB tubulointerstitial acute renal allograft rejection and Banff IIA vascular acute allograft rejection, however whether these findings may account for tissue damage and clinical signs observed during acute allograft renal rejection needs to be further studied.

References

1. *Chareandee C, Herman WH, Hrcik DE, Simonson MS:* Elevated endothelin-1 in tubular epithelium is associated with renal allograft rejection. Am J Kidney Dis 2000, 36, 541-549.

2. *Cornet S, Braillon A, Guilmard C, Chabrier P-E, Pirrotzky E, Braquet P:* Involvement of endothelin in renal processes. Hypertension 1990, 15, 724-728.

3. *Deng DX, Jiang J, Garcia B, Zhong R, Chakrabarti S*: Endothelin-1, endothelin-3 and their receptors endothelin(A) and endothelin(B) in chronic renal transplant rejection in rats. Transpl Int 2000, 13, 175-182.

4. *Eddy AA:* Molecular insights into renal interstitial fibrosis. J Am Soc Nephrol 1996, 7, 2495-2508.

5. *Herman WH, Emancipator SN, Rhoten RLP, Simonson MS:* Vascular and glomerular expression of endothelin-1 in normal kidney. Am J Physiol 1998, 275, F8-F17.

6. *Imai T, Hirata Y, Emori T, Yanagisawa M, Masaki T, Marumo F:* Induction of endothelin-1 gene by angiotensin and vasopressin in endothelial cells. Hypertension 1992, 19, 753-757.

7. *Karret FE, Davenport AP:* Localization of endothelin peptides in human kidney. Kidney Int 1996, 49, 382-387.

8. *Kiowski W, Luscher TF, Lindner L, Buchler FR:* Endothelin-1-induced vasoconstriction in humans. Reversal by calcium channel blockade, but not by nitrovasodilatators or endothelium-derived relaxing factor. Circulation 1991, 83, 469-475.

9. *Kohan DE:* Endothelins in the kidney: physiology and pathophysiology. Am J Kidney Dis 1993, 22, 493-510.

10. *Kurichara H, Yoshizumi M, Sugiyama T, Takaku F, Yanagisawa M, Masaki T, Hamaoki M, Kato H, Yazaki Y:* Transforming growth factor-β stimulates the expression of endothelin mRNA by vascular endothelial cells. Biochem Biophys Commun 1989, 159, 1435-1440.

11. *Luscher TF, Boulanger CM, Dohi Y, Yang ZH:* Endothelium-derived contracting factors. Hypertension 1992, 19, 117-130.

12. *Luscher TF, Wenzel RR:* Endothelin in renal disease: role of endothelin antagonists. Nephrol Dial Transplant 1995, 10, 162-166.

13. *Nagano H, Nadeau KC, Takada M, Kusaka M, Tilney MN:* Sequential cellular and molecular kinetics in acutely rejecting renal allografts in rats. Transplantation 1997, 63, 1101-1108.

14. *Naicker S, Gathiram P, Naidoo S, Nadar A, Muller-Esterl W, Bhoola KD:* Endothelin-1 and endothelin receptor status in kidney transplants undergoing acute rejection. Immunopharmacology 1999, 44, 67-74.

15. *Omatsu S, Tomoyoshi T:* Immunohistochemical study on endothelin in rat kidney. Hinyokika Kiyo 1997, 43, 109-114.

16. *Ong ACM, Jowett TP, Firth JD, Burton S, Karet FE, Fine LG:* An endothelin-1 mediated autocrine growth loop involved in human renal tubular regeneration. Kidney Int 1995, 43, 390-401.

17. *Rabelink TJ, Kaasjager KAH, Boer P, Stroes EG, Braam B, Koomans HA:* Effects of endothelin-1 on renal function in humans: Implications for physiology and pathophysiology. Kidney Int 1994, 46, 376-381.

18. *Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, Croker BP, Demetris AJ, Drachenberg CB, Fogo AB, Furness P, Gaber LW, Gibson IW, Glotz D, Goldberg JC, Grande J, Halloran PF, Hansen HE, Hartley B, Hayry PJ, Hill CM, Hoffman EO, Hunsicker LG, Lindblad AS, Marcussen N, Mihatsch MJ, Nadasdy T, Nickerson P, Olsen TS, Papadimitriou JC, Randhawa PS, Rayner DC, Roberts I, Rose S, Rush D, Salinas-Madrigal L, Salomon DR, Sund S, Taskinen E, Trpkov K, Yamaguchi Y:* The Banff 97 working classification of renal allograft pathology. Kidney Int 1999, 55, 713-723.

19. *Ruschitzka F, Shaw S, Gygi D, Noll G, Barton M, Luschner TF:* Endothelial dysfunction in acute renal failure. J Am Soc Nephrol 1999, 10, 953-962.

20. *Sakurai T, Yanagisawa M, Takuwa Y, Miyazaki H, Kimura S, Goto K, Masaki T:* Cloning of cDNA encoding a non-isopeptide-selective subtype of endothelin receptor. Nature 1990, 348, 732-735.

21. *Simonson MS:* Endothelins: Multifunctional renal peptides. Physiol Rev 1993, 73, 375-411.

22. *Simonson MS, Emancipator SN, Knauss T, Hricik DE:* Elevated neointimal endothelin-1 in transplantation-associated arteriosclerosis of renal allograft recipients. Kidney Int 1998, 54, 960-971.

23. *Sorensen SS, Madsen JK, Pedersen EB:* Systemic and renal effect of intravenous infusion of endothelin-1 in healthy human volunteers. Am J Physiol 1994, 266, F411-F418.

24. *Watschinger B, Vychtil A, Attar M, Wagner D, Schuller M, Hartter E, Ulrich W:* Pattern of endothelin immunostaining during rejection episodes after kidney transplantation. Clin Nephrol 1994, 41, 86-93.

25. *Yanagisawa M, Kurihara H, Kimura S, Tomobe Y, Kobayashi M, Mitsui Y, Yazaki Y, Goto K, Masaki T:* A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 1988, 332, 411-415.

26. *Yoshizumi M, Kurihara H, Morita T, Yamashita T, Oh-hashi Y, Sugiyama T, Takaku F, Yanagisawa M, Masaki T, Yazaki Y:* Interleukin-1 increases the production of endothelin-1 by cultured endothelial cells. Biochem Biophys Res Commun 1990, 166, 324-329.

Address for correspondence and reprint requests to:
M. Wągrowska-Danilewicz M.D., Ph.D.
Department of Nephropathology, Medical University of Łódź
Czechosłowacka 8/10, 92-216 Łódź