THE CO-LOCALIZATION OF TISSUE KALLIKREIN AND TRANSFORMING GROWTH FACTOR-β1 IN THE NON-CANCEROUS AND CANCEROUS HUMAN KIDNEY

BY

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DEDICATION

This dissertation is dedicated in honour of my late father Bobby Moodley, I thank you for:

'Being the special father you were and more so, your endless love that stretched so far. Always being there, for your care, and the special and true understanding we shared. The times you listened and for words not said, when words could not mend. The screaming and shouting to get through, and for all of those times that I needed you. Standing through the years by my side and also for the comfort when tears I could not hide. Knowing and not knowing what to do, and for easing and pulling me through. And I thank you most especially, for being my father.'

PREFACE

This study represents original work by the author and has not been submitted in any form to another university or institute. Where use was made of the work of others it has been duly acknowledged in the text.

The research described in this dissertation was carried out at the Department of Biotechnology, M.L. Sultan Campus, Durban Institute of Technology, under the supervision of Prof. KD Bhoola and Prof. B Odhav.

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ABBREVIATIONS

1. ADH	-	Antidiuretic hormone
2. AP	-	Alkaline Phosphatase
3. BC	-	Parietal epithelial cells of Bowman's capsule
4. BK	-	Bradykinin
5. CPN	-	Carboxypeptidase N
6. DAB	-	3,3' Diaminobenzidine
7. DCT	-	Distal convoluted tubule
8. DT	-	Distal tubular cells
9. EGF	-	Epidermal Growth Factor
10. FAST 1	-	Forkhead Activin-induced Signal Transducer
11. FGF	-	Fibroblast Growth Factor
12. GFR	-	Glomerular filtration rate
13. GM	-	Glomerular Mesangial Cells
14. GM	-	Glomerular mesangial cells
15. H_2O_2	-	Peroxidase
16. HGF	-	Hepatocyte Growth Factor
17. HK	-	High molecular weight kininogen; H-kininogen
18. ICC	-	Immunocytochemistry
19. IGF	-	Insulin-like Growth Factor
20. IGF-I	-	Insulin-like Growth Factor Receptor I
21. IGF-II	-	Insulin-like Growth Factor Receptor II
22. IgG	-	Immunoglobulin
23. IL-1	-	Interleukin-1
24. JGA	-	Juxtaglomerular apparatus
25. KI	-	Kininase I carboxypeptidases
26. KI-CPN	-	Kininase I-Carboxypeptidase N
27. KI-CPM	-	Kininase I-Carboxypeptidase M
28. KII	-	Kininase II carboxypeptidases
29. KII-ACE	-	Kininase II-Angiotensin I Converting Enzyme

30. KII-NEP	-	Kininase II-Neutral Endopeptidase
31. KKS	-	Kallikrein-kinin system
32. KLK	-	Tissue kallikrein multigene family
33. KLK1	-	Mammalian Tissue kallikrein
34. LAB-SA	-	Labelled-[strept] Avidin-biotin
35. LAP	-	Latent Associated Peptide
36. LK	-	Low molecular weight kininogen; L-kininogen
37. LTGF-β	-	Latent Transforming Growth Factor-beta
38. MAP	-	Mitogen-Activated Protein
39. NEP	-	Neutral Endopeptidase
40. NO	-	Nitric oxide
41. PAP	-	Peroxidase-Antiperoxidase
42. PBS	-	Phosphate Buffered Saline
43. PDGF	-	Platelet-derived Growth Factor
44. PG	-	Prostaglandin
45. PI-3-K	-	Phosphatidylinositol-3-OH Kinase
46. PK	-	Plasma kallikrein
47. PT	-	Proximal tubular cells
48. RBF	-	Renal blood flow
49. RCC	-	Renal cell carcinoma
50. rhTGF-β1	-	Recombinant Human Transforming Growth Factor-beta 1
51. RMIC	-	Renal medullary interstitial cells
52. RT	-	Room Temperature
53. rTK	-	Recombinant Tissue Kallikrein
54. SARA	-	Smad Anchor for Receptor Activation
55. SMADS	-	Transforming growth factor receptor substrate
56. TBRI	-	Transforming growth factor-beta Receptor type I
57. TBRII	-	Transforming growth factor-beta Receptor type II
58. TGF-β	-	Transforming Growth Factor-Beta
59. TGF-α	-	Transforming Growth Factor-alpha
60. TK	-	Tissue kallikrein

61. VEGF	-	Vascular Endothelial Growth Factor
62. VEGFR-I	-	Vascular Endothelial Growth Factor Receptor I
63. VEGFR-II	-	Vascular Endothelial Growth Factor Receptor II

ABSRACT

Evidence suggests that the induction of tissue kallikrein, and the subsequently formed kinins, enhances proliferation of tumour cells because of their mitogenic property. Additionally, the kinin peptides are believed to promote the invasion of normal tissue by tumour cells. TGF-β1 is a potent inhibitor of the growth of renal epithelial cells, and is a classical anti-mitogen, which is central to many of its antiproliferative effects. No studies thus far have been performed, as to whether the proposed anti-mitogenesis of TGF-β1 has a regulatory effect on the cell proliferative action of kinins on renal epithelial and carcinoma cells. As a first step therefore, experiments were designed to localize tissue kallikrein, kinin receptors B1 and B2, TGF-β1 and the TGF-β receptors II and III in non-cancerous and cancerous human kidney. Subsequent experiments examined cellular orientation of tissue kallikrein and TGF-β1 in the human renal epithelial and carcinoma cells.

To achieve these objectives, the following experiments were designed. Three-micrometer human cancerous and non-cancerous kidney wax sections adhered to Poly-L-lysine coated slides were cut. These sections were subsequently labelled by single-immunnolabelling to localize individual antigens. Antibodies to tissue kallikrein, kinin receptors B1 and B2, TGF-β1 and TGF-β receptors II and III were immunolocalized in control (non-cancerous) and cancerous human kidney using the peroxidase-antiperoxidase method for single-immunolabelling experiments. A double-staining technique using the LAB-SA method was performed to co-localize tissue kallikrein and TGF-β1 in the non-cancerous and cancerous human kidney tissue. Sections were subsequently mounted and photographed using X20 magnification.

The results showed that tissue kallikrein was localized predominantly in the distal tubule cells, with some clear labelling in the proximal tubule cells of the cancerous kidney tissue samples. The proximal localisation of tissue kallikrein in the non-cancerous renal tissue is due probably to glomerular disease, which finding is at variance with the tissue kallikrein localisation in the normal kidney (see page 64).

In the non-cancerous kidney, kinin receptor B1 was localized to the distal and proximal tubular cells, but in contrast there was increase in labelling at these sites, with inconsistent labelling of the parietal epithelial cells of Bowman's capsule in the cancerous kidney tissue. Kinin B2 receptor was localized to the distal and proximal tubular cells, glomerular mesangial cells and parietal epithelial cells of Bowman's capsule in the non-cancerous kidney, with an overall increase in labelling intensity at these sites in the cancerous kidney. TGF-β1 was localized to the distal tubular cells, proximal tubular cells, the glomerular mesangial cells and the parietal epithelial cells of Bowman's capsule in both non-cancerous and cancerous kidney with an increase in intensity of the labelling in the cancerous kidney. In the non-cancerous kidney both the distal and proximal tubular cells labelled positively TGF-β receptor II. There was an increase in labelling of the distal tubular cells of the cancerous kidney. TGF-β receptor III showed labelling in the distal tubular cells only in the non-cancerous kidney. There was increased labelling in the distal and proximal tubule cells of the cancerous kidney tissue. Using double-immunolabelling, tissue kallikrein and TGF-β1 were co-localized in the distal and proximal tubular cells of the non-cancerous and cancerous kidney. These results confirmed the single-immunolabelling results of tissue kallikrein and TGF-β1.

The results of this study showed that tissue kallikrein and TGF-β1 do co-localize in the human kidney, and hence we hypothesize that TGF-β1 may regulate the expression of tissue kallikrein, and therefore because of its anti-mitogenic property the proliferative and mitogenic actions of kinins on cancer cell.

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

The renal system is the principle organ system responsible for water and electrolyte homeostasis, providing the mechanism by which excess water and electrolytes are eliminated from the body. A second major function of the renal system is the excretion of many toxic metabolic waste products. Since all body fluids are maintained in dynamic equilibrium with one another via the circulatory system, any adjustment in the composition of the blood is reflected in complementary changes in the other fluid compartments of the body. Thus regulation of the osmotic concentration of blood plasma ensures the osmotic regulation of all other body fluids.

The gross structure of the kidney reflects the arrangement of nephrons within it (Fig. 1.1). The substance of the kidney may be divided into an outer cortex and an inner medulla. The major portion of each nephron is located in the cortex. The medulla is arranged into pyramid-shaped units called medullary pyramids. These convey ducts that converge to discharge urine at their apices. The nephron, the functional unit of the kidney, consists two major components, the renal corpuscle and the renal tubule.

The renal corpuscle is that part of the nephron responsible for the filtration of plasma and is a combination of two structures, Bowman's capsule and a tightly coiled network of capillaries, the glomerulus. In the renal corpuscle, elements of plasma are filtered from the glomerular capillaries into Bowman's space, and the glomerular filtrate then passes into the renal tubule. The renal tubule extends from Bowman's capsule. The primary function of the renal tubule is the selective reabsorption of water, inorganic ions and other molecules from the glomerular filtrate. In addition, some inorganic ions are secreted directly from blood into the lumen of the tubule. The highly convoluted renal tubule has four distinct histo-physiological zones (Fig. 1.2), each of which has a different role in tubular function.

a) The proximal convoluted tubule (PCT) makes up bulk of the renal cortex. Approximately 75% of all the ions and water of the glomerular filtrate are reabsorbed from the PCT. This reabsorptive function is reflected in the structure of the epithelial lining. The simple, tall and cuboidal epithelium has a prominent brush border, which almost completely fills the lumen. The brush border greatly increases the surface area of plasma membrane through which molecules can be reabsorbed from the glomerular filtrate. Molecules reabsorbed from the glomerular filtrate are returned back into the circulation via the capillary network arising from the efferent arteriole.
b) The loop of Henle, which functions in generating a high osmotic pressure in the extracellular fluid of the renal medulla.
c) The distal convoluted tubule (DCT) in which sodium ions are actively reabsorbed. This process is controlled by the adreno-cortical hormone, aldosterone. Sodium reabsorption is in some way coupled with the secretion of hydrogen and potassium ions into the tubular fluid. One hydrogen ion or one potassium ion is secreted for every one sodium ion reabsorbed. The most striking difference compared to the PCT, is that it lacks a brush border, having only a few irregular microvilli at the luminal surface. The DCT has less cytoplasm and the nuclei sit closer to the luminal surface and consequently tend to bulge into the lumen.
d) The collecting tubule is the terminal portion of the DCT, and streams tubular fluid into the collecting ducts. The collecting tubules and ducts are not normally permeable to water, however, in some instances there may be conditions, which may influence the reabsorption of water in the collecting tubules, for instance, in the presence of antidiuretic hormone (ADH).

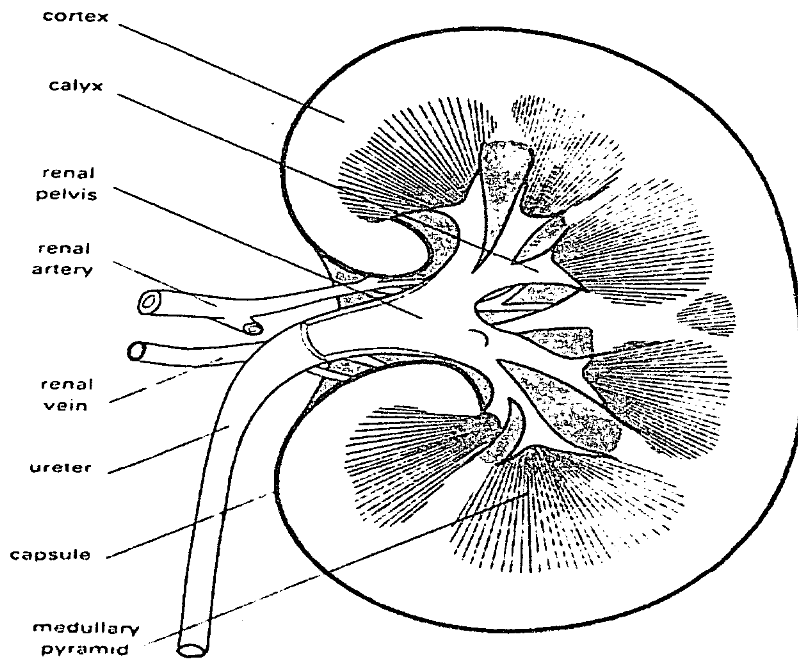

Fig.1.1 Gross structure of the kidney (Young and Heath, 2000)

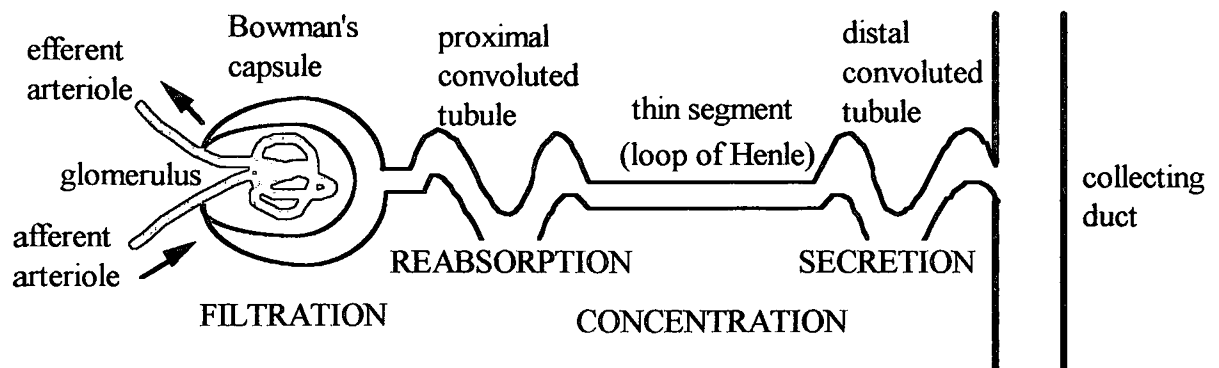

Fig.1.2 Diagrammatic structure of the nephron illustrating the four histo-physiological zones

The kidneys play an important role in the control of water and electrolyte balance, and the way it exerts this function is through interactions with either circulating or locally produced hormones e.g. renin, angiotensin, aldosterone and vasoactive peptides such as bradykinin (BK). Tissue kallikrein (TK) is a serine protease enzyme that acts on kininogen to form the biologically active kinins (BK and lysyl-BK [kallidin]). The binding of kinin with B2 receptors produces a broad spectrum of biological effects, including smooth muscle contraction and relaxation, increase in vascular permeability, vasodilation, electrolyte and glucose transport, and pain. Renal kallikrein and kininogen are localized in the connecting segment of the DCT and cortical collecting tubules. Release of kallikrein into the tubular fluid and interstitium can be stimulated by prostaglandins (PG), mineralocorticoids, angiotensin II, high potassium ions and diuretics. Kinin B1 and B2 are the two major receptors that exert most of the actions of kinins. Although the glomerulus and the distal nephron segments contain both kinin B1 and B2 receptors, most of the renal vascular and tubular effects appear to be mediated by B2-receptor activation. BK and kallidin elicit vasodilation and stimulate nitric oxide, PG E2 (PGE2) and I2 (PGI2), and renin release. The kallikrein-kinin system (KKS) is stimulated by sodium depletion, indicating it serves as a mechanism to dampen or offset the effects of angiotensin II levels.

The development of the kidney is a complex process involving many interdependent events. Polypeptide growth factors are essential for the normal development of the kidney and constitute a potent class of extracellular and/or intracellular signal molecules in regulating cellular proliferation and differentiation. The transforming growth factors-β (TGF-β) are widely recognised as a family of morphogenetic growth factors with regulatory roles in cell growth, differentiation, and immune function. TGF-β has a wide variety of biological effects and plays important although poorly understood roles in the normal structure and glomerular and tubular function. TGF-β1 has been best investigated among all three TGF-β isoforms, and is responsible for controlling the growth and differentiation of renal epithelial cells as well as tissue regeneration. TGF-β1 regulates diverse cellular processes by binding to three high-affinity cell surface receptors (types I,

II and III). In the normal kidney, TGF-β1 and its receptors have been localised to the glomeruli and tubules.

Cancer of the kidney is a disease in which the cells in certain tissues of the kidney start to grow uncontrollably and form tumours. Several types of cancer can afflict the kidneys:

a) Renal cell carcinoma (RCC), the most common form, accounts for approximately 85% of all kidney cancers. In RCC, malignant (cancerous) cells develop in the lining of the kidney's tubules that filter and clean the blood and typically grow into a mass called a tumour. Single tumours are the norm, although more than one tumour can develop within one or both kidneys.
b) About 7% of kidney cancers begin not in the kidney itself, but in the renal pelvis, the point where the kidney joins the ureter. These tumours are called transitional cell carcinomas, and are made up of cancer cells different from those that characterise RCC. Research indicates that cigarette smoke is an aetiological factor in these tumours.
c) A relatively rare form of kidney cancer, Wilm's tumour (also known as nephroblastoma) accounts for about 5 to 8% of kidney tumours in children. It occurs in about 7 out of 1 million children around the world per year, regardless of race, and is thought to be caused by genetic mutation that causes abnormal growth within the tubules of the kidney. The disease occurs equally in boys and girls. It typically first appears in children between 2 and 5 years of age, but has been known to occur rarely in adolescents as old as 15. Wilm's tumour can arise anywhere within the kidney's tissues. Untreated, it can spread, invading veins, lymph nodes, the adrenal glands, large or small bowel and liver.
d) Another rare form of kidney cancer, renal sarcoma is a disease of the kidney's connective tissues that accounts for less than 1% of all kidney tumours. Its symptoms are similar to those of RCC: haematuria, pain in the back or flank, or a lump or mass in the abdomen. In most cases, it is impossible to differentiate renal sarcoma from RCC externally, so the diagnosis usually is made after examination of a CT scan or MRI procedure. Such tumours will grow and spread to adjacent organs, bones and lymph nodes if left untreated.

Previous studies on renal carcinoma have immunovisualised the TK-kinin cascade and TGFB system in the glomeruli, proximal and distal tubules. TK is expressed in response to pathological stimuli, and the mitogenic property of the formed kinins enhances the proliferation of tumour cells. TGF-β1 suppresses malignancy in TGF-β1-responsive epithelial cancer cells, and therefore may be viewed as a negative growth factor. To examine critically their spatial relationships and orientation, the study was focussed on the localization of TK, kinin B1 and B2 receptors, TGF-β1 and TGF-β receptor II and receptor III, in order to gain insights on their role, if any, in kidney cancer. Experiments using antibodies directed to TK, kinin B1 and B2 receptors, TGF-β1 and TGF-β receptor II and receptor III using immunolocalization studies were conducted. The co-localization of TK and TGF-β1 was also determined to ascertain their relationship in kidney cancer.

The literature search (Chapter One) covers the kallikrein-kinin system and the transforming growth factor-β system and other growth regulatory proteins. Since this is the first study investigating the co-occurrence of TK and TGF-β1, no literature relating to this aspect is included.

The materials and methods (Chapter Three) describes the methodology used for localizing TK, kinin B1 and B2 receptors, TGF-β1 and TGF-β receptor II and receptor III in kidney cells using the peroxidase-antiperoxidase method. This is followed by the methodology used to determine the co-localization of TK and TGF-β1. The results of these studies are depicted in Chapter Four which is followed by the discussion (Chapter Five) and conclusion (Chapter Six) of this study.

1.2 LITERATURE REVIEW

1.2.1 THE KALLIKREIN-KININ SYSTEM

Kallikreins belong to a sub-group of the serine protease family and may be responsible for the post-translational processing of protein precursors. By means of enzymic action, kallikreins release the vasoactive peptides, kinins, from endogenous substrates called kininogens (Muller-Esterl, 1989). Enzymes that possess the capacity to release kinins from kininogens are collectively called kininogenases. The enzymes are further subdivided into plasma and glandular (commonly termed tissue) kallikreins. Distribution of the kallikreins, either in an active and/or inactive form, range from various glandular cells and neutrophils to urine and biological fluids (Bhoola *et al.*, 1992b). These kininogenases are two distinct entities that differ from each other with respect to their molecular weights, isoelectric points, substrate specificities, immunological profiles, the type of kinin released and functional importance (Bhoola *et al.*, 1992a); with TK being able to process low molecular weight kininogen (LK) to produce kallidin (lys-BK), and plasma kallikrein (PK) liberating BK from high molecular weight kininogen (HK) (Fig.1.3, Bhoola *et al.*, 1992b).

Regulatory processes, such as organ perfusion, systemic blood pressure, sodium and water homeostasis, regulation and maturation of growth factors, and inflammation, are regulated by the humorally generated kinins (Chen *et al.*, 1988; Yu *et al.*, 1998). The kinin peptides liberated from kininogens by the enzymic action of kallikreins are active biological peptides that participate in a wide range of physiological effects. Kinins exert their action via two known receptors namely, kinin B1 and B2 receptors, which modulate their cellular actions. The physiological relevance of each component of the KKS continues to unfold with important advances.

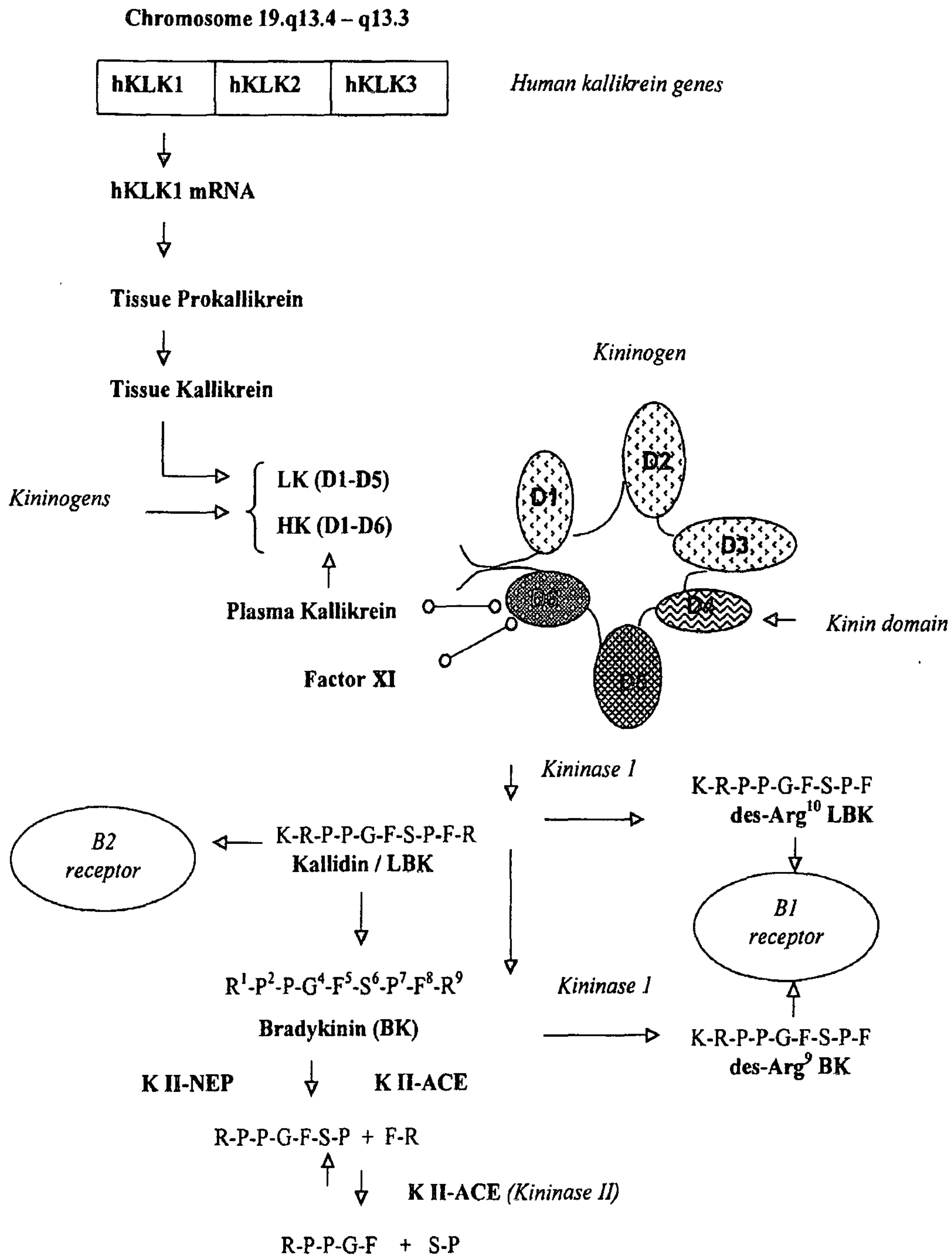

Fig 1.3 Overview of the KKS (Bhoola *et al.*, 2001)

1.2.1.1 Tissue Kallikrein

TK, otherwise known as glandular kallikrein, was first discovered in urine as a hypotensive material. TK are acid glycoproteins, which unlike trypsin, have a high specificity for polypeptide substrate cleavage. TK is encoded by a multigene (KLK) family in several species (Murray *et al.*, 1990). The true mammalian tissue kallikrein (EC.3.4.21.35), encoded by the gene KLK1 (Fig 1.3), has been classified a member of a subdivision of serine proteases able to release kinins by hydrolytic activity on one methionyl and one arginyl bond on the physiological substrate kininogen, thereby liberating the decapeptide kallidin.

The initially discovered three genes of the human family code for proteins with either trypsin-like or chymo-trypsin-like activity. All genes do not show kininogenase activity but have extensive homologues at DNA and protein levels (Bhoola *et al.*, 1992b). They are all clustered on human chromosome 19q13.3-q13.4. Estimates of the size of the human kallikrein gene differs from 3-4-19 kbs, suggesting that the human kallikrein gene may be much larger than previously thought (Clements, 1994). Southern blot analysis indicates that there may be as many as 19 human KLK genes (Murray *et al.*, 1990). Yousef and Diamandis (2000) have defined a 300 kb human kallikrein gene region on chromosome 19q13.3-13.4. They have expanded the human KLK family with several newly identified serine proteases.

Eleven new kallikrein genes are recognised, all clustered within the 300 kb region. These new kallikrein genes have several features in common, they are localised to the same chromosomal site, they encode for serine proteases with the conserved catalytic triad (histidine, aspartic acid and serine); all have five coding exons which are similar, they have significant sequence homologies and many are regulated by steroid hormones. All kallikrein genes are 5-7 kb in size and comprise five exons and four introns (Rittenhouse *et al.*, 1998). The three originally described kallikrein genes have the classical 'kallikrein loop' – the loop region is thought to be important for substrate activity (Clements, 1997). The newer described genes do not have this loop, indicating that there has been more

divergence in humans than in rodents. The first three genes show good sequence and protein homology between the newer genes especially those situated telomerically from KLK1-3 (Anisowicz *et al.*, 1996; Liu *et al.*, 1996; Yousef *et al.*, 1999; Yousef and Diamandis 1999; 2000).

The human TK genes are clustered on the long arm of chromosome 19. Salivary gland kallikrein DNA using southern blot analysis was hybridised against mouse/human hybrid cell lines using a panel of chromosomes, 1, 3, 4, 7, 8, 10, 12, 13, 16, 17, 20, 21, 22, 11 and 14. None or a weak signal was seen (Sutherland *et al.*, 1988). The strongest signal was observed on the long arm of chromosome 19, indicating that the location of kallikrein gene is at or close to the 19qter – region of 19q13.2-19q13.4. The finding of one region suggests that all the human kallikrein genes may be localised in one single locus (Evans *et al.*, 1988).

The expression patterns of the kallikrein genes show prostate restricted expression (KLK2-4) or pancreas-restricted expression (KLK6-13). Lower expression is noted in kidney, ovary, testis, and salivary gland (Harvey *et al.*, 2000). KLK14 showed high expression in brain/bone marrow. These genes appear clustered together on the chromosome. These genes also show variable expression in cancer cell lines. Gene induction of TK and the subsequently formed kinins enhances the proliferation of tumour cells (Rae *et al.*, 1999). New kallikrein-like genes KLK-L2 and KLK-L1 are upregulated by oestrogens and other hormones in the breast cancer cell line BT-474 (Yousef and Diamandis, 1999; Yousef *et al.*, 1999) while KLK-L4 is downregulated in breast cancer (Yousef *et al.*, 2000) and NES1 in breast and possibly other cancers (Diamandis *et al.*, 2000).

1.2.1.2 Kallistatin

The actions of proteases are regulated, in part, by the presence of specific inhibitors. It has since been established that kallikrein inhibitors in plasma, serum and other biological fluids are high molecular weight proteins able to occlude or bind catalytic sites of kallikrein (Worthy *et al.*, 1990). A member of the serine proteinase inhibitor family - kallistatin or human TK-binding protein has been identified, purified and cloned (Chao *et al.*, 1986; 1995; 1996). Human kallistatin is located on chromosome 14q31-32.1 (Chai *et al.*, 1994). The human kallistatin gene is a single-copy gene. Kallistatins are acidic glycoproteins with molecular masses of 58-60 kDa and they form a reversible complex with kallikrein (target enzyme).

Human kallistatin and TK are co-localised in a variety of tissues. The major site is the liver, with lower expression in the pancreas and kidney. Expression and cellular localisation of this inhibitor have been identified in various human tissues, cells and body fluids (Chai *et al.*, 1993; Chen *et al.*, 1995; Chao *et al.*, 1995). The mRNA of this protein is expressed in the inner medullary ducts of the kidney with small amounts in the outer medullary collecting duct, PCT and the glomerulus. This strengthens the role of the intrarenal KKS for a paracrine function in renal homeostasis. The expression of kallikrein-binding proteins appear to be regulated by hormones such as oestrogen, progesterone, growth hormone and thyroxin. Expression is reduced during acute inflammation, ischaemic strokes and in hypertensive rats.

1.2.1.3 Kininogens

The primary physiological substrate for kallikreins are kininogens. Kininogens are the large multifunctional proteins that contain the kinin sequence (Kato *et al.*, 1988; Muller-Esterl *et al.*, 1986). Three types of kininogens have thus far been described, the largest being a H-kininogen form found in blood, a L-kininogen form found in different tissues and blood (Worthy *et al.*, 1990), and T-kininogen unique to the rat. Mammalian

kininogens (HK and LK) are products of a single gene (Takagaki *et al.*, 1985). This single kininogen gene (K gene), codes for bovine and human kininogens. The gene has been localised on chromosome 3q26→qter (Fong *et al.*, 1991; Cheung *et al.*, 1992).

Kininogen is synthesized by the hepatocytes. H-kininogen, (HK), a glycoprotein that is a non-enzymic co-factor in the coagulation mechanism, is contained and secreted by unstimulated and activated platelets (Schmaier *et al.*, 1986). L-kininogen (LK) is cleaved by TK and has been co-localised to sites of TK action, for example vascular smooth muscle cells in the mouse.

Kininogen has been detected in human urine. Immunoreactive kininogen was localised in the principal cells of the collecting duct and restricted to the luminal portion of the principal cells (Figueroa *et al.*, 1988). The close relationship between the cells containing TK and its substrate, kininogen, suggests that kinins could be generated in the lumen of the collecting tubules. The mRNA of LK is expressed in the renal cortex and medulla, suggesting the biosynthesis of LK in the distal tubule.

1.2.1.4 Kinins

Kinins are potent bioactive peptides formed by the enzymic action of kininogenases on kininogen, and are thought to be involved in inflammation, pain generation, and regulation of blood pressure (Figueroa *et al.*, 1990). Kinins are the vasoactive peptides that signal the biological effects of the KKS. The two most potent kininogenases are PK and TK They generate kinins, in various tissue and biological fluids by enzymatic action. PK acts on HK to produce the nonapeptide BK (Werle *et al.*, 1961), and tissue/glandular kallikrein releases the decapeptide kallidin (lys-BK) from LK (Fiedler, 1979), which is rapidly converted to BK by the action of arginine aminopeptidase. Some conversion of kallidin to BK may also occur. Other kinins such as T-kinin and met F-kinin have been described so far, in the rat. Earlier reports included the discovery of a kinin in the venom sacs of hornets (Bhoola and Schacter, 1960). Mammalian kinins exhibit a highly

conserved structure, and there is a remarkable degree of homology between the insect, reptile and amphibian kinins. In blood, the half-life of BK and kallidin is estimated to be less than 30 seconds because of rapid degradation by peptidases called kininases, but may not be so short-lived in other biological fluids and in tissue spaces.

The two mammalian kinins, kallidin (lys-BK) and BK, influence the cardinal features of inflammation as well as a number of cellular functions, including blood pressure and local blood flow, electrolyte and glucose transport, and cell proliferation. New and more extended functions have been described to kinins, and are thought to be specific and are considered to regulate local blood flow and ion movement in the kidney. Both H and L-kininogens have been shown to be present in the collecting ducts of the distal nephron (Hermann *et al.*, 1996). This allows for the generation of kinins in the distal tubule, where the connecting duct meets the collecting ducts. High concentrations of free kinins have been found in the terminal portions of the distal nephron, confirming this cellular orientation of enzyme and substrate. The action of kinins is therefore related to the distal tubule where much of renal homeostasis is said to take place.

Kinins also stimulate the secretion of renin from the kidney, release vasopressin from the neurohypophysis and secretion of catecholamines from the adrenal medulla. The ability of kinins to induce cell division could enhance the spread of cancerous cells and increase proliferation of epidermal cells in disorders. Kinins also appear to play an important role in a number of pathological states, namely, reduced sperm motility, allergic and viral rhinitis and asthma, inflammatory bowel disease and carcinoid.

1.2.1.5 Kinin Receptors

Kinins express their pharmacological effects by activating specific kinin receptors situated on the surface membranes of many cell types (Schanstra *et al.*, 1998). Thus far, two major subtypes of the kinin receptor, designated B1 and B2 have been identified, which are linked to specific G-protein-coupled second messengers. The existence of

other subtypes has not reached a clear-cut conclusion - the existence of a B3 receptor (Farmer *et al.*, 1989) was proposed following studies in the guinea-pig trachea. The receptors modulate the cellular actions of kinins, and are present in low copy number in most natural cells (Hall and Morton, 1997; Blaukat *et al.*, 1999).

Kinin B1 Receptors

Kinin B1 receptors were originally described in rabbit aorta (Steranka and Burch, 1991). They have now been documented in cultured cells of vascular, endothelial, mesangial, tracheal, bone and fibroblast origin. The kinin B1 receptor does not seem to be readily expressed in normal tissue, but appears to be induced in chronic inflammatory processes (Menke *et al.*, 1994). The kinin B1 receptor gene was cloned from a human genomic library, and is encoded by a single copy gene (Bachvarov *et al.*, 1996). The gene is located on chromosome 14q32.1-q32.2 in close proximity to the kinin B2 receptor gene (Chai *et al.*, 1996). The kinin B1 receptor has a predicted sequence of 353 amino acids and shows 36% protein sequence homology and 54% nucleotide homology with the kinin B2 receptor.

The kinin B1 receptor is not constitutively expressed but is induced in response to inflammation over a period of time. This has been confirmed by *in vitro* studies on vascular tissue (Butt *et al.*, 1995), and in non-vascular tissue (Boschcov *et al.*, 1984). Several growth factors such as epidermal growth factors (EGF) and endothelial growth factor also induce a kinin B1 receptor response (Boutillier *et al.*, 1987). Induction of the kinin B1 receptor involves de novo protein synthesis and this synthesis may be induced by the products of tissue damage or noxious agents (Regoli *et al.*, 1978). *In vitro* studies have demonstrated a link between inflammatory mediators and kinin B1 receptors (Farmer *et al.*, 1991). Endogenously produced cytokines have been implicated in mechanisms of inflammation and hyperalgesia in both animals (Colditz and Watson, 1992) and in man (Remick *et al.*, 1992). Both interleukin-1 (IL-1) and interleukin-2 (IL-2) have been shown to increase the rate of kinin responses (De Blois *et al.*, 1988).

B1 receptors are restricted in their localisation, and have been demonstrated predominantly in vascular beds. It has been established that kinin B1 receptors are not constitutively expressed in normal renal cells but during inflammation and tumour growth, there is a marked up-regulation of kinin B1 receptors. The sites include, the distal tubular cells, proximal tubular cells, parietal epithelial cells of Bowman's capsule and to a lesser degree the mesangial cells. They have also been expressed in a number of cultured cell lines, bovine pulmonary artery (Cahill *et al.*, 1988), embryonic mice osteoblast cells (Ljunggren and Lerner, 1990), human fibroblasts (Goldstein and Wall, 1984) and rabbit fibroblasts (Marceau and Temblay, 1986).

Kinin B2 Receptors

Kinin B2 receptors have been described in numerous sites, and the majority of the *in vivo* effects of kinins are mediated by the activation of B2 receptors (Steranka and Burch, 1991; Dray and Perkins, 1993). The gene for the human kinin B2 receptor was isolated from human placenta genomic library. The gene is located on chromosome 14q32 (Powell *et al.*, 1993; Kammerer *et al.*, 1995). The rat and mouse receptors have a protein sequence of 366 amino acids and the human receptor has a predicted sequence of 364 amino acids. The kinin B2 receptor is widely distributed with highest concentration in the kidney. It is also localised in the heart, lung, brain, uterus, testes, vessels and neutrophils (McEachern *et al.*, 1991; Bascands *et al.*, 1993; Haaseman *et al.*, 1994; Song *et al.*, 1996;).

In the rat kidney, the B2 receptor has been found in the straight portions of the proximal tubules, distal straight tubules, connecting tubules and collecting ducts (Figueroa *et al.*, 1992b). The B2 receptors are present in the luminal membranes, basal infoldings of the tubular cells and in smooth muscle cells of the cortical radial artery and afferent arterioles. The B2 receptors are co-localised with TK and kininogen in connecting tubules and collecting ducts cells respectively. B2 binding sites have been ultrastructurally mapped to the vasa recta bundles, capillary endothelial cells, epithelial

cells of the thin limbs, distal tubule, collecting duct and renal medullary interstitial cells (RMIC) in the inner stripe of the outer medulla. In the inner medulla, B2 binding sites were localised to RMICs, loops of Henle, capillary endothelium and collecting duct epithelial cells.

1.2.1.6 Kinin Receptor Antagonists

Since kinins are potent inflammatory mediators, invoking both kinin B2 receptors in the early stage of disease and kinin B1 receptors in prolonged chronic conditions, antagonists to both receptor subtypes are targets of drug therapy. By definition, antagonists are compounds that bind to the receptors without invoking intrinsic activity. Production of antagonists involves long, painstaking searches for analogues and then chemical modification of analogue structure, to produce an antagonistic effect. Following the discovery of the biochemical structure for BK, many analogues were synthesised.

The first antagonist to inhibit the physiological actions of BK were produced by Vavrek and Stewart (1985). The initial peptide antagonists were rapidly metabolised and not selective for receptor subtype. The most useful first generation peptide antagonist was NPC-349, increasing its affinity for kinin receptors. The compound was rapidly metabolised by carboxypeptidase N (CPN), kininase-1. The second-generation peptide antagonists followed this, the most stable being HOE-140 (Icatibant). This compound reacts predominantly with the kinin B2 receptor but with removal of the C-terminal arginine, proves to be a potent B1 antagonist.

Stewart and workers modified the HOE-140 antagonist by the addition of α-2-indanylglycine (Ig1) at position five and seven (Stewart and Gera, 1996; Stewart *et al.*, 1997) to produce B-9430 a third generation peptide antagonist. This compound is resistant to degradation by neutral endopeptidase (NEP)-kininase. This antagonism is effective for both kinin B2 and B1 receptors, although it does contain the C-terminal arginine residue (Hanson *et al.*, 1996). However with removal of the C-terminal

arginine, this compound becomes selectively a B1 antagonist (B-9858). These compounds are also effective in various species and in humans (Burkard *et al.*, 1996).

HOE-140 has been the most widely used kinin B2 receptor antagonist. It shows high affinity in most species, but more so to the rat and human B2 receptor. It exerts a competitive antagonism on the human B2 receptor (Marceau *et al.*, 1994); but not on other B2 receptors of other species (Regoli *et al.*, 1993), where it acts as a non-equilibrium antagonist. Its use in pathology has been to evaluate the role of kinins in pain and hyperalgesia (Dray and Perkins, 1993), in inflammation (Griesbacher and Lembeck, 1992).

Although the kinin B1 receptor antagonists were discovered first, their use has been limited because the role of the B1 receptor in inflammation was not recognised. The first antagonist [Leu^8] des-Arg^9-BK had the C-terminal Phe residue replaced by Leu and this almost completely eliminated the biological action at kinin B2 receptors, (Regoli *et al.*, 1977). They have been the most useful for characterisation of the kinin B1 receptors in inflammation and pain (Dray and Perkins, 1993).

All of the antagonists currently in use are peptide analogs. Their use is limited because of their poor oral bioavailability. The search for newer more potent nonpeptide receptor antagonists has produced the first orally active kinin B2 receptor antagonist, FR173657 (Asano *et al.*, 1997; Inamura *et al.*, 1997). This antagonist shows high potency in many animal species, rabbit, guinea pig, mouse and human. This antagonist is also specific for the kinin B2 receptor, and is inactive against kinin B1 receptor (Asano *et al.*, 1999). Nonpeptide kinin B1 receptor antagonists are not available for clinical use at present, but the active site of the B1 receptor is small enough to bind nonpeptide compounds (similar to angiotensin AT1 receptor); there is belief therefore that this type of antagonists will soon be available. Both types of antagonists will be useful to research and therapeutic medicine.

1.2.1.7 Kininases

The turnover of kinins depends on both the rate of formation and the rate of destruction. After kinins are formed, they are rapidly destroyed by the enzymatic action of peptidases. The sites at which they cleave kinins are indicated in figure 1.3. This family of enzymes is generally called kininases (Erdos, 1990). There are two families of kininases, KI (Kininase I carboxypeptidases) and KII (Kininase II peptidylpeptidases). The KI family comprises KI-CPN (Kininase I-Carboxypeptidase N) and KI-CPM (Kininase I-Carboxypeptidase M) and the KII family includes KII-ACE (Kininase II-Angiotensin I-Converting Enzyme) and KII-NEP (Kininase II-Neutral Endopeptidase).

KI-CPN is an arginine carboxypeptidase which is optimally active at pH 7.4 and activated by cobalt chloride ($CoCl_2$). This enzyme acts on COOH-terminal end of the BK molecule and by removing the Arg^9 residue produces a kinin B1 receptor antagonist. It is synthesized by the liver and secreted into the circulation where it probably accounts for about 90% of the BK-destroying activity in human plasma (Zacest *et al.*, 1974). It has been found in placenta, kidney, lung and pulmonary arterial endothelial cells (Johnson *et al.*, 1984). It is distributed in two major portions of the nephron: in the proximal tubules and the medullary collecting ducts. KI-CPM is a 62 kDa metallopeptidase activated by cobalt. The molecule shows only 41% identity with the active subunit of KI-CPN (Bhoola *et al.*, 1992b). It has been found in placenta, kidney, lung and pulmonary arterial endothelial cells (Johnson *et al.*, 1984).

KII-ACE inactivates circulating BK and at the same time converts angiotensin I to angiotensin II by the removal of the COOH-terminal His-Leu in the pulmonary circulation. KII-ACE is found as a soluble enzyme in biological fluids, vascular endothelium, neuroepithelial cells, brush border of the choriod plexus, microvilli of the placenta, small intestine. Kininase II is concentrated along the brush border membrane of proximal tubule cells and the S3 segments of the proximal tubules (Bhoola *et al.*, 1992b).

NEP accounts for more than half of the renal kininases in humans, while accounting for 68% of the total kininase activity in rat urine, with kininase II and kininase I accounting for 23% and 9% respectively. NEP is present in the outer surface of the brush border of proximal tubules and to a lesser extent, in the vesicular organelles in the apical cytoplasm and basal infoldings of the proximal tubule cells (Bhoola *et al.*, 1992b).

1.2.2 GROWTH FACTORS – CELL REGULATORY PROTEINS

Over the years considerable data have accumulated on the molecular processes by which the cell phenotype is determined. The discovery of polypeptide growth factors and the subsequent scientific advances, places them on cutting edge of research. Peptide growth factors are regulatory proteins that govern the response of the cell to injury and mediate the highly co-ordinated processes of cell growth, differentiation and death (apoptosis). Peptide growth factors may use either autocrine or paracrine pathways to signal cells in the microenvironment. A new concept of autocrine and paracrine action was formulated when observations on the topographical distribution of the growth factors in developing and adult tissues were compared to their effects on cell growth, cell differentiation, cell-cell and cell-matrix interaction. Thus, the growth factors are believed mainly to exert their effects in an autocrine (cell expressing both the growth factor and its receptor) and paracrine (different adjacent cell type expressing either the growth factor or its receptor) and do so usually by binding to high affinity plasma membrane receptors that transduce a signal via an intracellular cascade of activation reactions involving phosphorylations. Because of the exquisite sensitivity of cells to these polypeptide effector molecules, the regulation of their action is controlled at many levels, including gene transcription and translation, receptor activation, and intracellular signalling (Sporn and Roberts, 1992). An additional mechanism controlling growth factor action is the regulation of their extracellular availability via the initial secretion of inactive forms that are either latent or sequestered (Rifkin, 1997).

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In contrast to the endocrine mode of action of the classical hormones, most growth factors are secreted and act at short range (Rifkin *et al.*, 1999). As a mechanism to control extracellular growth factor action and to insure that these molecules display a regulated function in time and space, they circulate latent molecules (Rifkin, 1997). Proteases also appear to play a fundamental role in the mobilisation of growth factors.

1.2.2.1 Transforming growth factor-β superfamily

The TGF-β superfamily is one of the most complex groups of cytokines with widespread effects on many aspects of growth and development. The TGF-β isoforms and other family members, have diverse effects in similar physiological situations. The TGF-β superfamily gene expresses a large set of structurally and functionally related polypeptides. Because of the recent rapid increase in the number of different growth factors of the TGF-β superfamily, the boundaries between the subfamilies have become less clear. Thus far, five distinct TGF-β isoforms have been characterised that share 64 to 82% homology among their amino acid sequences (Roberts *et al.*, 1991). The different isoforms of TGF-β have been cloned from various sources, including TGF-βs 1, 2 and 3 which are regulated by specific genes and have been identified in mammals (Derynck *et al.*, 1985), chicken TGF-β4 (Jakowlew *et al.*, 1988) and *Xenopus* TGF-β5 (Kondaiah *et al.*, 1990). The three protein isoforms identified in the mammal are abundantly observed during development and display overlapping and distinct spatial and temporal patterns of expression. Each isoform plays a distinct role, the nature of which depends on the cell type, its state of differentiation and the growth conditions and on the other growth factors present.

The active forms of TGF-β are homodimers with each monomer having a molecular mass of 12 500d, except TGF-β4. Heterodimers such as TGF-β1.2, which consists on one monomer from TGF-β2, do exist in porcine platelets (Cheifetz *et al.*, 1987). Each

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monomer or subunit has nine cysteines, which are common to all the five isoforms (Rocco and Ziyadeh, 1991; Daopin *et al.*, 1992; Massague, 1992). The larger superfamily of TGF-β like proteins, include activins, inhibins, Mullerian-inhibiting substance, and the bone morphogenetic proteins (Massague, 1990). These proteins have about 30% amino acid sequence identity and share seven invariant cysteines (Daopin *et al.*, 1992). The members of TGF-β superfamily have very diverse and profound effects on various stages of development as well as maintaining tissue function and integrity during adult life.

1.2.2.2 TGF-β

Current interest in TGF-β is very different from the context in which this peptide was originally discovered. Like many other peptide growth factors, TGF-β is multifunctional, and many of its most important activities have little to do with the transformation system in which it was first discovered. Biological actions of TGF-β *in vitro* are very diverse. Much of the interest in TGF-β reflects its importance as a mediator of inflammation, repair, and angiogenesis, as well as its importance as a negative growth regulator for many epithelial cells and for both T and B lymphocytes. It has been shown to stimulate cellular proliferation *in vitro*, but is generally considered to be an inhibitor of proliferation and a promoter of cellular hypertrophy and differentiation (Roberts and Speared, 1990; Choi *et al.*, 1993). TGF-β has also been shown to block or initiate cellular differentiation and cell migration depending upon the cell type and culture conditions (Roberts and Speared, 1990). The inhibitory action is quite broad, targeting epithelial, endothelial as well as hematopoeitic cells. Because essentially all cells have receptors for TGF-β, TGF-β has the potential to regulate physiological function in almost all tissues of the body.

Mature TGF-β is noncovalently associated with the 80 kD latency associated peptide (LAP). LAP is a fundamental component of TGF-β and is required for its efficient secretion, prevents it from binding to ubiquitous cell surface receptors and maintains its availability in a large extracellular reservoir that is readily accessed by activation. This latent TGF-β complex (LTGF-β) is secreted by all cells and is abundant both in

circulating forms and bound to the extracellular matrix. In the normal kidney, it is reported to be present in glomeruli, but not in tubules or interstitium whereas under pathological conditions, tubular epithelial cells express this protein (Yanagida *et al.*, 1994). Activation describes the collective events leading to the release of TGF-β (Gleizes *et al.*, 1996; Munger *et al.*, 1997). The deposition of TGF-β in the extracellular matrix in a latent form has wide biological implications. TGF-β itself is a multipotent growth factor, which employs the unique postsecretory latency concept. The LTGF-β-binding proteins are required for the correct folding and secretion of TGF-β and target TGF-β to extracellular structures. The accumulation of extracellular microfibrillar structures ensure that the whole TGF-β system is capable of providing focussed and fast responses via activation of the stored growth factor (Saharinen and Keski-Oja, 2000).

1.2.2.2.1 TGF-β1

Human TGF-β was first discovered from placenta (Frolik *et al.*, 1983) and platelets (Assoian *et al.*, 1983), and the TGF-β1 gene was cloned in 1985 (Derynck *et al.*, 1985). TGF-β1 is the prototype of its highly homologous isoforms TGF-β2 and TGF-β3 as well as a superfamily of over 40 different related proteins, presumed to have been derived from a common ancestor gene (Roberts and Speared, 1990; Kingsley, 1994). The active TGF-β1 molecule is a 25 kDa homodimer of two 12.5 kDa disulphide linked monomers. Each monomer is derived as the carboxy terminal of a 390 amino acid precursor molecule, and this structure is shared by the other isoforms. TGF-β1 appears to play the major role, both quantitatively and qualitatively.

The overall sequence homology between human TGF-β1, β2, and β3 is about 75%, reflecting a strong conservation between the sequences encoding the mature (active) TGF-β sequence, and a more relaxed homology between the precursor sequences (Kingsley, 1994). The genes for TGF-β1, β2, and β3 are located on chromosomes 19q13, 1q44 and 14q24, respectively (Massague, 1990). The human TGF-β1 precursor is

encoded by seven exons. The positions of the intron/exon junctions of TGF-β1 are conserved in TGF-β2 and TGF-β3, with the exception of the first which differs by three nucleotides, which suggests that the various TGF-βs originated from duplication of a common ancestral gene (Derynk *et al.*, 1988). The TGF-β mRNA species range from 1.7 to 6.5 kb with extensions at both the 3' and 5' ends of the coding sequence and these extensions are quite distinctive for the different TGF-βs; the noncoding regions of TGF-β1 are very G-C rich, whereas those of TGF-β2 are relatively A-T rich, with those of the other TGF-βs falling between these two extremes. TGF-β1 has been best investigated among all three TGF-β isoforms, and it is thought that TGF-β1 plays an important role in pathological extracellular matrix accumulation found in glomerulosclerosis and interstitial fibrosis (Border and Noble, 1994). However, the expression of TGF-β in normal kidney suggests its involvement in normal physiologic renal function.

1.2.2.2.2 Expression and regulation of TGF-β

TGF-β is expressed in virtually all human tissues, the richest source being platelets. No general pattern of expression of TGF-β exists. Many human malignant cell types have retained the expression patterns of their normal counterparts, but others show alterations in their TGF-β expression, which in some instances are thought to contribute to their malignant properties.

The expression of TGF-β is regulated by a number of different factors. Autoinduction, the induction of expression of TGF-β, in response to TGF-β treatment of the cells, was shown in different cell types (Kelley *et al.*, 1993). In addition, TGF-β expression in various cell types is regulated by hormones, other growth factors and phorbol esters. The regulation of TGF-β expression can take place at the transcriptional level. The promoter for TGF-β lacks the classic TATAA box but has multiple regulatory sites that can be activated by immediate early genes like *c-jun*, *c-fos*, *egr-1*; by a number of oncogenes like *abl*, *fos*, *jun*, *ras* and *src* (Birchenall-Roberts *et al.*, 1990; Kim *et al.*, 1990). Activity

of the TGF-β1 promoter is suppressed by tumor supressors including the products of the retinoblastoma susceptibility gene and the Wilm's tumor gene (Dey *et al.*, 1994).

1.2.2.2.3 TGF-β receptors

Virtually every cell in the body, including epithelial, endothelial, haemapoietic, neuronal and connective tissue cells, produce TGF-β1 and has receptors for it. TGF-β1 regulates cellular processes by binding to three high-affinity cell surface receptors known as types I, type II and type III receptors (Massague and Like, 1985; Cheifetz *et al.*, 1987). They all bind TGF-β with high affinity. A wide range of normal and malignant cell types express these receptor proteins. In the kidney, TGF-β type I, II and III receptors were found in the glomeruli and renal tubules (Ando *et al.*, 1998).

TGF-β Receptor-I

The TGF-β receptor-I has distinctive biochemical properties (Cheifetz and Massague, 1991), but its primary structure is not known. The type I receptors are glycoproteins with molecular weights of approximately 53 kDa and is present in almost all nontransformed cells (Sharma and Ziyadeh, 1993). From studies it was proposed that the type I receptor only binds TGF-β, when co-expressed with a type II receptor (Laiho *et al.*, 1991). Moreover, expression of the type I receptor is mandatory for TGF-β to exert its growth inhibitory effect in these cells (Boyd and Massague, 1989). An interesting feature of some of the type I receptor isoforms is that they are able to bind either TGF-β or activin depending on whether they are co-expressed with the TGF-β type II receptor or the activin type II receptor, respectively (Attisano *et al.*, 1993; Ebner *et al.*, 1993; Tsuchida *et al.*, 1993; ten Dijke *et al.*, 1994). This functional diversity has only been demonstrated for isoforms without signalling capacity and the physiological relevance of this phenomenon is still to be identified.

TGF-β Receptor-II

Analogous to the type I receptors, the type II receptors for different members of the TGF-β superfamily are structurally related. In contrast they show a lower degree of sequence similarity than the type I receptors, and do not bind other members of the superfamily (Lin *et al.*, 1992; Kingsley, 1994). Like TGF-β type I receptors, the type II receptor is a single transmembrane glycoprotein with a serine/threonine kinase domain in the cytoplasmic part. The receptor is slightly larger 70 to 85 kDa - than type I receptor. Only one form of TGF-β type II receptor has been identified.

TGF-β Receptor-III

Betaglycan (type III receptor) is a 280 to 330 kDa proteoglycan consisting of a 110 kDa core protein with a variable number of glycan side-chains (Andres *et al.*, 1991) and a short cytoplasmic tail with no obvious signalling motif. The human betaglycan (Moren *et al.*, 1992) and its rat and porcine homologs (Lopez-Casillas *et al.*, 1991) have been cloned and characterised and were found to have features different from the type I and II receptors. Betaglycan is ubiquitously expressed along with type I and type II receptors, but usually in a considerably higher number, thus providing the majority of TGF-β binding sites on most cells (Damstrup *et al.*, 1993). The expression of betaglycan is dispensable for mediation of the different cellular responses to TGF-β. However, the presence of betaglycan increases the binding of TGF-β to the type II (signalling) receptor through the formation of a complex between the two receptors, leading to increased biological response to TGF-β. Therefore it was proposed that the role betaglycan was that of a ligand capacitor, presenting TGF-β to the signalling receptors.

1.2.2.2.4 TGF-β signalling

Biologic signals for TGF-β are transduced through heteromeric complexes of TGF-β type I and II receptors. These receptors act in concert to activate signalling. Upon binding to TGF-β, the type II receptor (TβRII) forms a heteromeric complex with the type I receptor (TβRI), resulting in the phosphorylation and activation of the TβRI (Wrana *et al.*, 1994). The activated TβRI then interacts with an adaptor protein SARA (smad anchor for receptor activation) that propagates signals to several transcription factors known as Smads (Blobe *et al.*, 2000; Miyazono, 2000; Datto and Wang, 2000). Smads can be divided into three classes based on their functional properties, the receptor-regulated Smads (Smad1, 2, 3, 5, 8), the common Smads (Smad4 and 4β) and the antagonistic Smads (Smad6 and 7).

Studies of the TGF-β signalling pathway have shown that upon activation of TβRI, Smad2 and Smad3 transiently associate with the receptor and are directly phosphorylated by the receptor kinase (Heldin *et al.*, 1997; Massague, 1998). The phosphorylated Smad then forms a heteromeric complex with Smad4 and this complex translocates from the cytoplasm to the nucleus, where they activate specific target genes through co-operative interactions with DNA and DNA-binding proteins such as FAST1 and Fos/Jun(AP-1) (Zhang *et al.*, 1998) (Fig. 1.4). By contrast, Smad6 and Smad7 interfere with the activation of the effector Smads and act as 'inhibitory' Smads. Both Smad6 and Smad7 can interact with TβRI, thus competitively preventing the 'receptor-activated' Smads from being phosphorylated.

The receptor-activated Smads are linked to the general transcriptional machinery through a direct physical interaction with the transcriptional coactivator CBP/p300, which is essential for the transcriptional activation function of Smads (Janknecht *et al.*, 1998; Shen, 1998). The transcriptional activation of Smads, through their physical interactions and functional cooperativity with transcription factors, allows cross-talk with other signalling pathways (Zhang and Derynck, 1999; Massague, 2000). For example, the activation of mitogen-activated protein (MAP) kinase pathways, which is commonly

observed in tumor cells, and of Jun kinase or p38 MAP kinase is likely to regulate TGF-β-induced transcription from promoters with TGF-β-responsive AP-1 (c-Jun/c-Fos)- or CREB/ATF-binding sites and Smad-binding sites (Zhang *et al.*, 1998; Hanafusa, 1999; Wong, 1999).

Most notably, the expression of extracellular matrix proteins and proteases in response to TGF-β often requires an intact AP-1-binding promoter sequence, suggesting that there is transcriptional cooperation of Smads with the AP-1 complex and a dependence of these TGF-β responses on Ras/MAP kinase and or phosphatidylinositol-3-OH kinase (PI-3-K) signalling (Wong, 1999). Ras/MAP kinase signalling also induces expression of TGF-β1, which can be enhanced further by TGF-β signalling and thus may explain the often-observed increase in expression of TGF-β1 by tumour cells. The effects of TGF-β receptor activation can be profoundly affected in the presence of other proteins that may be constitutively expressed in specific cells types. Cells that express SnoN and Ski may be relatively unresponsive to TGF-β stimulation. In certain cells, ligand activation of the TGF-β receptor may cause the rapid degradation of pre-existing SnoN, resulting in the removal of an important obstacle to TGF-β-mediated transcriptional activation. In yet other cells SnoN may persist after TGF-β receptor activation and thereby succeed in blocking an important component of the TGF-β signalling cascade involving Smad3/4 transcription factors (Sun *et al.*, 1999).

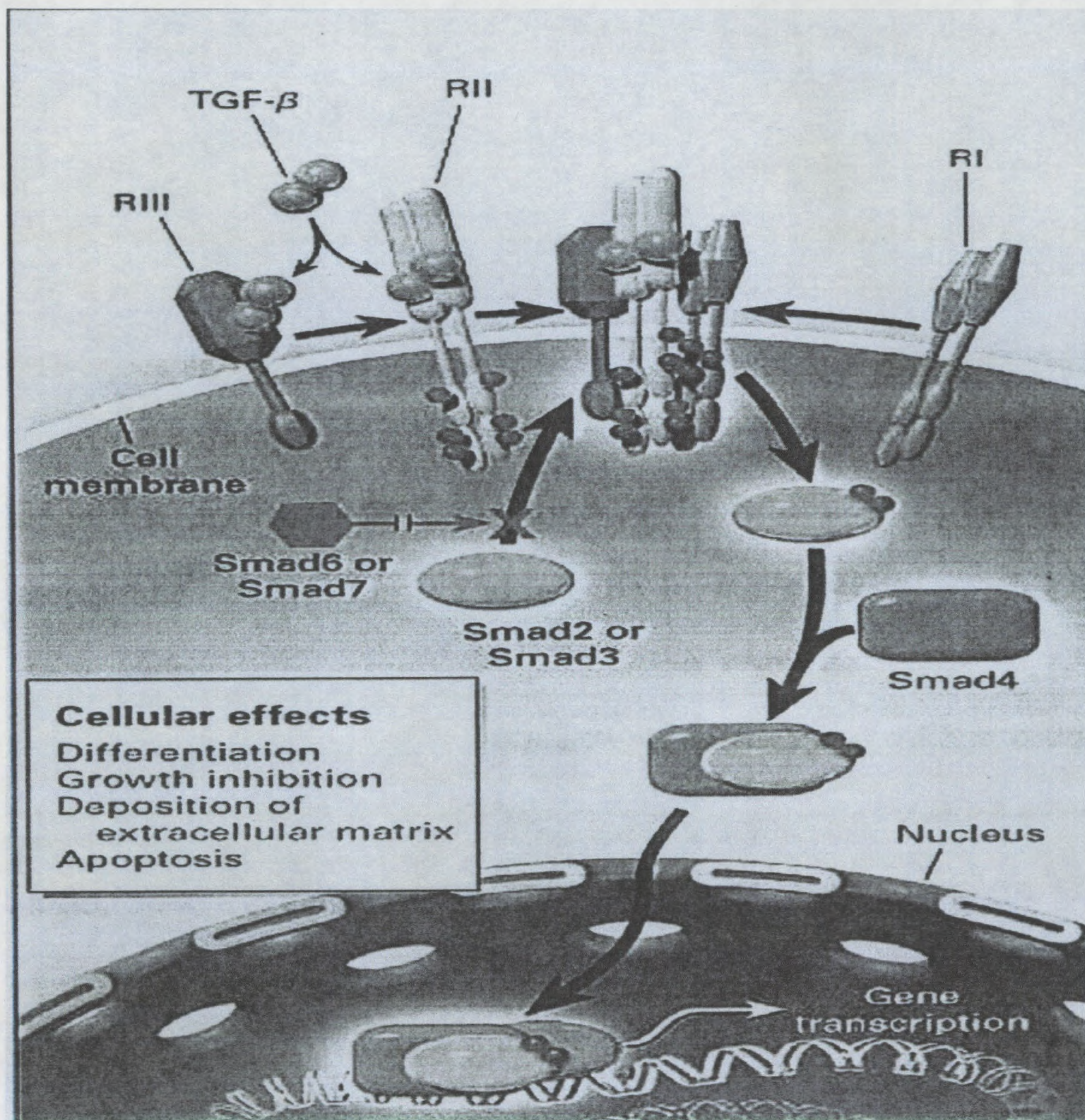

Fig.1.4 Mechanism of signal transduction mediated by TGF-β1

In the extracelluar space TGF-β1 binds either to type III TGF-β receptor (RIII), which presents it to the type II receptor (RII), or directly to RII on the cell membrane. The binding of TGF-β to RII then leads to binding of the type I receptor (RI) to the complex and the phosphorylation of RI. This activates the RI protein kinase, which then phosphorylates the transcription factor Smad 2 or 3. phosphorylated Smad 2 or 3 binds to Smad 4, and the resulting complex moves from the cytoplasm to the nucleus. In the nucleus, the Smad complex interacts in a cell specific manner with various other transcription factors to regulate the transcription of TGF-β-responsive genes and mediate the effects of TGF-β at the cellular level. Smad 6 and 7 block the phosphorylation of Smad 2 or 3, thus inhibiting TGF-β signalling (Blobe *et al.*, 2000).

1.2.2.3 Epidermal Growth Factor

The family of EGF-like peptides is currently considered, to include epidermal, transforming (alpha), amphiregulan, heparin binding growth factors and cripto. All of these molecules signal through the same EGF receptor, which is a 170 kD transmembrane glycoprotein linked to a tyrosine kinase (Carpenter and Cohen, 1990; Massague, 1990). EGF receptor binds and is activated by EGF, TGF-alpha (TGF-α), heparin-binding like-EGF, amphiregulan, and epiregulin (Moghal and Sternberg, 1999). EGF is a 53 kD multi-potential growth factor which promotes proliferation, migration, collagen production, and tubulogenesis *in vitro* (Fine *et al.*, 1992). It is also involved in embryogenesis, cellular differentiation and angiogenesis. In the kidney, EGF is expressed in thick ascending limbs, early DCT, and glomerular endothelial cells and the EGF receptors are abundantly distributed along the nephron.

1.2.2.4 Hepatocyte Growth Factor

Hepatocyte growth factor (HGF) is a multifunctional growth factor and has mitogenic, anti-apoptotic, and morphogenic activities on renal tubular cells, whereas it has angiogenic and angioprotective actions on endothelial cells (Matsumoto and Nakamura, 2001). HGF is synthesized from mesenchymal cells such as fibroblasts, endothelial cells, renal mesangial cells and macrophages. The HGF receptor, c-Met, is expressed in renal tubular cells, vascular endothelial cells, glomerular endothelial cells, podocytes and mesangial cells.

1.2.2.5 Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF), a member of the family of heparin-binding growth factors, plays an important role in angiogenesis, anti-apoptosis, proliferation and development. It is a dimeric glycoprotein made from alternative splicing of VEGF

mRNA into five isoforms. VEGF is expressed on podocytes and on renal tubules of the outer medulla and medullary ray (Kang *et al.*, 2001). VEGF has four receptors (Zachary and Gliki, 2001): VEGFR-1, VEGFR-2, VEGF-3 and a docking co-receptor, neuropilin-1, enhances binding of $VEGF_{165}$ to VEGFR-2 (Whitaker *et al.*, 2001) Most biological functions of VEGF are mediated via VEGFR-2 in the adult rat. VEGF-1 receptors are exclusively expressed on endothelial cells of the glomerular capillary and VEGFR-2 is induced on the mesangial cells in proliferative glomerulonephritis (Thomas *et al.*, 2000).

1.2.2.6 Insulin-like Growth Factor

The insulin-like growth factor (IGF) family is made up of IGF-I and IGF-II, and relaxin, and it shares a high degree of functional and sequence homology with insulin and proinsulin (Iwamura *et al.*, 1993; Peehl *et al.*, 1993). All of these peptide growth factors may signal via the IGF-I and -II receptors, and insulin receptor. IGF uses paracrine and autocrine pathways to stimulate cellular proliferation of normal and malignant stromal, epithelial and neuroectodermal cells (Cohen *et al.*, 1991).

1.2.2.7 Platelet-Derived Growth Factor

Platelet-derived growth factor (PDGF) is a potent mitogen and chemo-attractant of the mesenchymal cells. The PDGF family contains four genes from PDGFA to PDGFD (Uutela *et al.*, 2001). The PDGF makes a dimer form, which binds to the dimerized receptor-$\alpha\alpha$, $\alpha\beta$, and $\beta\beta$. PDGF-AA, PDGF-AB, PDGF-CC bind to the $\alpha\alpha$-receptor, while PDGF-BB and PDGF-DD bind to the $\beta\beta$-receptor. In the kidney, PDGF plays an important role in nephrogenesis (Betsholtz and Raines, 1997).

1.2.2.8 Fibroblast Growth Factor

The fibroblast growth factor (FGF) family contains at least 7 members, including acidic and basic FGF, int-2, hst/KS3, FGF-5, and keratinocyte growth factor (Burgess and Maciag, 1989). All members of this family can signal through common receptors. Members of the FGF family are mitogenic for epithelial, mesenchymal and neuroectodermal cells. Basic FGF, a 146 amino acid polypeptide, is the prototypical member of the FGF family. It is a potent angiogenic factor stimulating endothelial cell proliferation, migration and differentiation to form neovasculature (Gospodarowicz, 1990). Basic FGF and TGF-β regulate remodelling of the extracellular matrix by modulating extracellular matrix proteases, and promoting the synthesis of collagen, fibronectin and proteoglycans.

CHAPTER TWO: RESEARCH DESIGN

2.1 Rationale/ Hypothesis

TGF-β1 and TK have been independently visualized in the kidney. TGF-β1 is a potent inhibitor of the growth of kidney epithelial cells and a classical antimitogen, which is probably central to many of its antiproliferative effects. The induction of TK and the subsequently formed kinins, in view of their mitogenic property, enhance the proliferation of tumour cells, and additionally promote the invasion of normal tissue. One functional question to answer is whether the proposed antimitogenesis of TGF-β1 has a regulatory effect on the cell proliferative action of kinins in renal carcinoma.

2.2 Aim

The co-localization of TK TGF-β1 in renal carcinoma to gain an insight into the biological interaction between TK and TGF-β1

2.3 Objective

2.3.1 To localize TK, Kinin B1 and B2 receptors, TGF-β1, Receptor II and Receptor III in non-cancerous and cancerous human kidney.

2.3.2 To co-localize TK and TGF-β1 in non-cancerous and cancerous human kidney.

2.3.3 To determine if there is relationship between the co-occurrence of TK and TGF-β1 in human kidney carcinoma

CHAPTER THREE: MATERIALS AND METHODS

3.1 Ethical Permission

Permission for the collection of control kidney samples from forensic post-mortems and for the use of diagnostic renal biopsy tissue for research purposes was obtained from the Ethics Committee of the University of Natal, Medical School. Informed consent for diagnostic renal biopsy was obtained from patients by the attending Nephrologist, and sequestered with the relevant hospital records.

3.2 Tissue Samples

Kidney: Control (non-carcinoma)

The diagnostic kidney tissue was obtained from patients who presented clinical symptoms of renal disease. On histological examination the tissue appeared to show minimal renal pathology (non-cancerous renal tissue). Forensic post-mortem renal tissue was obtained from patients who died of accidental causes unrelated to injury or apparently not known to have renal pathology (n=2).

Kidney: Cancerous

Renal biopsies were obtained from patients who presented clinical features of renal cancer (n=4). The biopsies were performed as part of routine diagnostic procedure, under ultrasound guidance, with a 20-gauge trucut needle. Where possible, two or three cores of tissue were obtained.

3.3 Tissue Processing for Immunocytochemistry

Prior to immunolabelling kidney tissues were fixed onto poly-L-lysine coatedslides and sectioned.

Fixation and Embedding

Kidney tissue was fixed in 5% formal saline (35% formaldehyde in 0.9% NaCl, diluted 1:7) for 2 h. Renal biopsies were subsequently dehydrated in absolute ethanol at descending ethanolic concentration and embedded in wax.

Coating of Slides: Poly-L-lysine

Poly-L-lysine (0.1%, w/v) (Sigma, St Louis) was diluted with distilled water (v/v, 1:10). The diluted solution was allowed to reach room temperature (RT) (18 to 26^0C) before use. Clean slides were placed in the diluted Poly-L-lysine solution for 5 min at RT. Slides were drained and dried in a 60^0C oven for 1 h or at RT overnight.

Sectioning

Three micrometers kidney wax sections were cut using sterile disposable blades on a microtome (Shandon M1R, CSIL, France), and fixed onto adhesive coated slides (Poly-L-lysine, Sigma, St. Louis). The sections on coated slides were baked on a hot plate at 60^0C for 10 min before dewaxing as outlined in Table 3.1.

3.4 Immunolocalization of TK, Receptors B1 and B2, TGF-β1 and TGF-β Receptors II And III in Control (Non-Cancerous Renal Tissue) and Renal Carcinoma

3.4.1 Primary Antibodies

Antibodies to TK, Kinin Receptors B1 and B2, TGF-β1 and TGF-β Receptors II and III in control (non-carcinoma) and cancerous kidney were localized using the Peroxidase-Antiperoxidase Method (Elias, 1989). The primary antibodies were obtained as described below.

Anti-human Tissue Kallikrein Antibodies (Anti-rTK)

Polyclonal anti-human TK antibody was raised in goat, directed against recombinant tissue kallikrein (rTK) generated in *E.coli* transfected with human TK cDNA (Rahn *et al.*, 1992). The rTK was supplied by Dr Michael Kemme (Institute for Biochemistry, Technical University of Darmstadt, Darmstadt, Germany).

Anti-B1 Receptor Antibody (Anti-B1)

The Anti-B1 receptor antibody was kindly supplied by Fred Hess (Merck Research Laboratories, R80 M-213, Rahway, NJ 07065) having been fully characterised for specificity. It is a polyclonal antibody directed to the following C-terminus synthetic peptide of the kinin B1 receptor (Il3-Ser-Ser-Ser-His-Arg-Lys-Glu-Ile-Phe-Gln-leu-Phe-Trp-Arg-Asn) (I-S-S-S-H-R-K-E-I-F-Q-L-F-W-R-N) was raised in rabbits.

Anti-B2 Receptor Antibody (Anti-B2)

The antibodies were kindly provided by Werner Muller-Esterl (Cardiovascular Group, Institute of Biochemistry II, University Hospital Frankfurt, Theodor-Stern-Kai 7, D-60590, Germany). Of the eight polyclonal antibodies, fully characterised for specificity, raised to synthetic peptides of the amino-terminal and loop regions encoded by the rat kinin B2 receptor cDNA, and based on the homogeneity between these regions with the

human receptor, only four were shown to react strongly with human epithelial cells and neutrophils (Haasemann *et al.*, 1994). Of these, antibodies directed to:

1. Leu-His-Lys-Thr-Asn-Cys-Thr-Val-Ala-Glu for intracellular domain one (ID1, LHK, 280) {L-H-K-T-N-C-T-V-A-E for ID1}
2. Asp-Arg-Tyr-Leu-Ala-Leu-Val-Lys-Thr-Met-Ser-Met-Gly-Arg-Met for intracellular domain two (ID2, DRY, 277) {D-R-Y-L-A-L-V-K-T-M-S-M-G-R-M for ID2}
3. Asp-Thr-Leu-Leu-Arg-Leu-Gly-Val-Leu-Ser-Gly-Cys for the fourth extracellular domain (ED3, DTL, 283) {D-T-L-L-R-L-G-V-L-S-G-C for ED4}

were combined and used for immunolabelling, as the match between the peptide sequences of these regions in the rat and human receptors is 80%, 100%, and 75% respectively.

Anti-human TGF-β1 Antibody (Anti-TGF-β1)

The antibody used was purchased from Sigma Chemicals (St Louis). Monoclonal anti-human TGF-β1 (IgG1 isotype) is purified from a mouse hybridoma. Recombinant, human TGF-β1 (rhTGF-β1) expressed in CHO cells and latent TGF-β1 was used as immunogen. The antibody is purified by Protein A affinity chromatography. Monoclonal anti-human TGF-β1 will neutralize the biological activity of TGF-β1 and TGF-β1.2. By ELISA, the antibody shows <5 % cross-reactivity with TGF-β1.2 and >1 % cross-reactivity with TGF-β2, TGF-β3, and TGF-β5.

Anti-human TGF-β Receptor II Antibody (Anti-TGF-β RII)

The antibody used was purchased from Sigma Chemicals (St Louis). Anti-human TGF-β receptor II is developed in goat using a recombinant human soluble TGF-β RII, expressed in NSO cells as immunogen. The antibody is purified using human TGF-β RII affinity chromatography. Anti-human TGF-β RII may be used to neutralise the bioactivity mediated by human TGF-β RII. By ELISA, the antibody shows no cross-reactivity with other cytokines.

Anti-human TGF-β Receptor III Antibody (Anti-TGF-β RIII)

The antibody used was purchased from Sigma Chemicals (St Louis). Anti-human TGF-β receptor III (betaglycan) is developed in goat using a recombinant human soluble TGF-β RIII, expressed in NSO cells as immunogen. The antibody is purified using human TGF-β RIII affinity chromatography. Anti-human TGF-β RIII recognises the extracellular domain of human TGF-β RIII. By ELISA, the antibody shows no cross-reactivity with other cytokines.

3.4.2 Antibody Titre Determination

The antibodies were diluted at different concentrations in 0.01 M phosphate buffered saline (PBS), pH 7.4, for TK, Kinin Receptors B1 and B2 (1:50; 1:100; 1:200) and for TGF-β1, TGF-β Receptors II and III (1:2; 1:5; 1:10) to determine the concentration that produced the desired staining intensity. The dilutions that produced the desired staining intensity for TK, Kinin Receptors B1 and B2 were 1:50 and for TGF-β1, TGF-β Receptors II and III were 1:2.

3.4.3 Single-immunolabelling: Peroxidase-antiperoxidase (PAP) Method

Three µm thick sections of wax-embedded kidney tissue were adhered onto adhesive coated (poly-L-lysine, Sigma Chemicals) slides. The peroxidase-anti peroxidase (PAP) method (Elias, 1989) was used for immunolabelling. The kidney wax sections (normal and cancerous) on glass slides were heated on a hot tray and were dewaxed in analytical grade xylene for 30 min, rehydration with graded ethanolic solutions (100%, 90%, 70% in distilled water, v/v) and distilled water as the final rehydrant. Subsequently, it was immersed in absolute methanol/3% H_2O_2 (v/v) for 20 min to quench endogenous tissue peroxidase activity. Antigen retrieval/unmasking was performed by boiling the tissue in a metal-salt solution, 0.1 M sodium-citrate pH 6.0 at varying microwave power (medium

high, medium, low) for 5 min each in a conventional microwave oven. The tissue was then allowed to cool by immersing in 0.01 M PBS. The tissue was subsequently incubated in skim milk (10% w/w in 0.01 M PBS) and non-immune serum (Zymed, 859643) for 20 min each. Incubation with the primary antibodies (100 μl) was performed at 4^{0}C overnight at the various working dilutions. The primary antibodies used for single-immunolocalization are detailed below:

Anti-TK:	polyclonal goat anti-human recombinant TK IgG [(diluted 1:50) with 0.01 M PBS (pH 7.4)].
Anti-B1:	polyclonal anti-human recombinant B1 IgG [(diluted 1:50) with 0.01 M PBS (pH 7.4)].
Anti-B2:	polyclonal anti-human recombinant B2 IgG [(diluted 1:50) with 0.01 M PBS (pH 7.4)].
Anti-TGF-β1:	monoclonal mouse anti-human recombinant TGF-β1 IgG [(diluted 1:2) with 0.01 M PBS (pH 7.4)].
Anti-TGF-β RII:	polyclonal goat anti-human recombinant TGF-β RII IgG [(diluted 1:2) with 0.01 M PBS (pH 7.4)].
Anti-TGF-β RIII:	polyclonal goat anti-human recombinant TGF-β RIII IgG [(diluted 1:2) with 0.01 M PBS (pH 7.4)].

The tissue was subsequently treated with a PAP-streptavidin-biotin conjugated system (Zymed Laboratories, San Francisco) according to the manufacturers instructions. This entailed incubating the tissue with a universal IgG-biotin link (Zymed, 859643) for 20 min at room temperature. Next, the tissue sections were incubated with a streptavidin-peroxidase conjugate (Zymed, 859643) for 20 min at RT. The labelled antibody bound to the PAP immuno-enzyme complex was visualised by incubating the sections, in the dark, for 10 min with liquid 3,3' diaminobenzidine (DAB) chromogen (Zymed, 859643), resulting in a visible precipitate. The sections were counterstained with Mayers Haematoxylin (Sigma, St Louis) for 2 min. Sections were then dehydrated by a reversal of the rehydration process from distilled water, through the increasingly concentrated ethanolic solutions (70%; 90%; 100%) into xylene, and finally mounted with a permanent

medium, Entellen (Merck, Germany). The stained sections were viewed by conventional light microscopy under a Nikon photo-microscope (Nikon, Japan) at X20 magnification.

All incubations were carried out in a moist chamber. Between incubations the sections were washed thoroughly by submerging the slides in 0.01 M PBS (pH 7.4) at two 3 min intervals. Tissue sections were not allowed to dry out at any time during the labelling process. Labelled slides were stored in the dark.

A summary of the immunostaining protocol for the different antibodies is outlined in Table 3.1.

3.4.4 Co-localization of TK and TGF-β1 using Double-Immunolabelling

The method of Vandesande (1983) using a double-staining technique to show two distinct antigens in a single tissue was used. The kidney wax sections (normal and cancerous) on glass slides were heated on a hot tray at 60^0C, followed by dewaxing in analytical grade xylene for 30 min, rehydration with graded ethanolic solutions (100%, 90%, 70% in distilled water, v/v) and distilled water as the final rehydrant. Subsequently, it was immersed in absolute methanol / 3% H_2O_2 (v/v) for 10 min each to quench endogenous tissue-peroxidase activity. Antigen retrieval/unmasking was performed by boiling the tissue in a metal-salt solution, 0.1 M sodium- citrate pH 6.0 at varying microwave power (medium high, medium, low) for 5 min each in a conventional microwave oven. The tissue was then immersed in cold 0.01 M PBS (pH 7.4) to cool. The tissue was incubated in skimmed milk (10% w/w in 0.01 M PBS) and non-immune serum (Zymed, 95999) for 20 min each. The primary antibodies used for double-immunolocalization are detailed below:

Anti-TK:	polyclonal goat anti-human recombinant TK IgG [(diluted 1:50) with 0.01 M PBS (pH 7.4)].
Anti-TGF-β1:	monoclonal mouse anti-human recombinant TGF-β1 IgG [(diluted 1:2) with 0.01 M PBS (pH 7.4)].

Incubation with 100 µl of the primary antibody 1 (TGF-β1) was performed at 4^0C overnight. The tissue was subsequently treated using the labelled-[strept]Avidin-biotin (LAB-SA) method (Zymed Laboratories, San Francisco) according to the manufacturers instructions. This entailed incubating the tissue with a universal IgG-biotin link (Zymed, 95999) for 10 min at RT. The tissue sections were then incubated with a streptavidin-alkaline phosphatase conjugate (Zymed, 95999) for 10 min at RT. The first primary antibody (Anti-TGF-β1) was visualised by incubating the sections, in the dark, for 10 min with a substrate-chromogen mixture (Zymed, 95999) for alkaline phosphatase (AP), resulting in a visible dark purple stain.

Incubation with 100 µl of the second primary antibody (Anti-TK) was performed at 4^0C overnight. The tissue was incubated with a universal IgG-biotin link (Zymed, 95999) for 10 min at RT. The tissue sections were then incubated with a streptavidin-peroxidase conjugate (Zymed, 95999) for 10 min at RT. The second antibody (Anti-TK) was visualised by incubating the sections, in the dark, for 10 min with a substrate-chromogen mixture (AEC) (Zymed, 95999), resulting in a visible intense red stain. The sections were counterstained with Mayers Haematoxylin (Sigma, St Louis) for 30 s. Clearmount (Zymed, 95999) was used to cover the specimen and heated at 60^0C for 15 to 30 min. The stained sections were viewed by conventional light microscopy under a Nikon photo-microscope (Nikon, Japan) at X20 magnification.

All incubations were carried out in a moist chamber. Between incubations the sections were washed thoroughly by submerging the slides in 0.01 M PBS (pH 7.4) at three 2 min intervals. Tissue sections were not allowed to dry out at any time during the labelling process. Labelled slides were stored in the dark.

A summary of the immuno-histological double-staining technique for the co-localization of TK and TGF-β1 is outlined in Table 3.2.

3.4.5 Positive Tissue and Method Controls for Immunocytochemistry (ICC)

Positive Controls

Sections of normal human kidney, served as positive control tissue (Fig 3.1a).

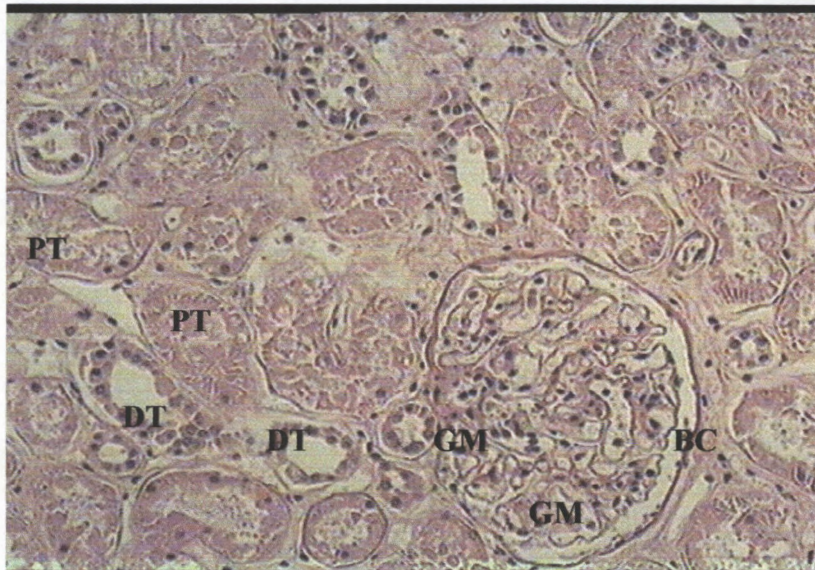

Fig 3.1a Haematoxylin and Eosin Staining of a normal human kidney section: photomicrograph of the renal cortex showing the glomerular mesangial cells (GM), distal (DT) and proximal (PT) tubular cells and parietal epithelial cells of Bowman's capsule (BC)

Method Controls

During each labelling run, the primary antibody on one tissue section was replaced with buffer/non-immune serum. Negative staining would be expected when one part of the

labelling "bridge" was removed, no positive labelling was obtained on these control sections. Examination of the renal tissue showed a typical glomerulus, proximal tubular cell with brush border, Bowman's capsule and distal tubular cells (Figure 3.1b). No labelling of the glomerulus or tubules was observed.

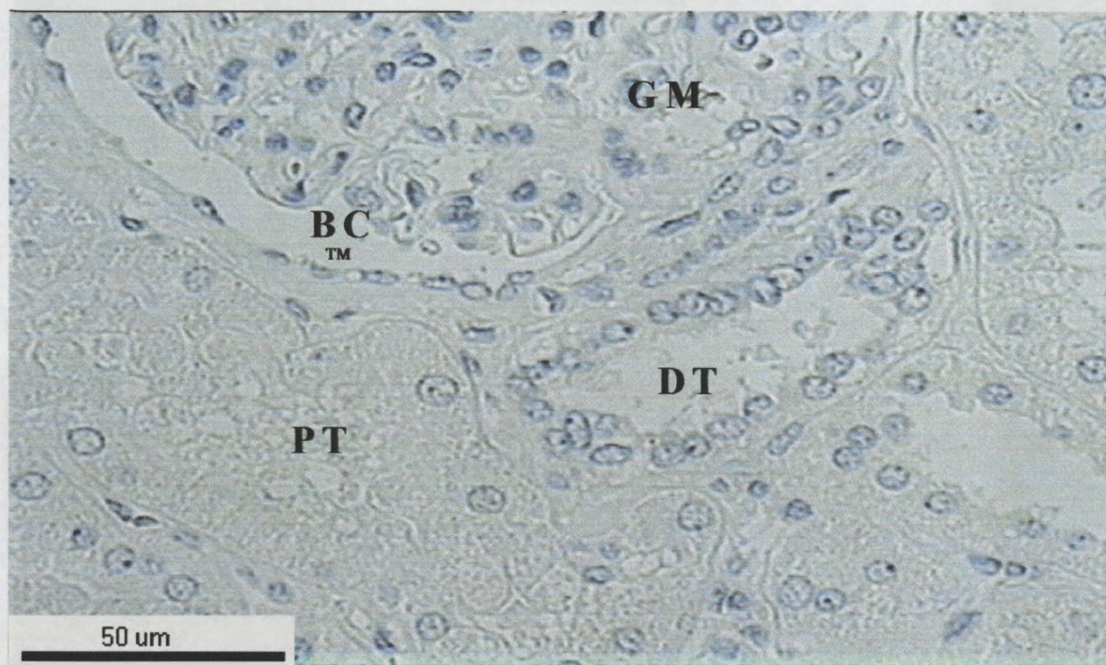

Fig 3.1b Method Control: photomicrograph of the renal cortex showing the glomerular mesangial cells (GM), distal (DT) and proximal (PT) tubular cells and parietal epithelial cells of Bowman's capsule (BC)

Table 3.1: Procedure for single-labeling immunohistochemical localization

Table 3.1: Procedure for single-labelling immunohistochemical localization

STEP	TREATMENT	TIME
1	**Dewax and Hydrate sections** Xylene 100 % EtOH 100 % MeOH + 3 % H_2O_2 90 % EtOH 70 % EtOH dH_2O	 30 min 2 x 3 min 2 x 10 min 2 x 3 min 2 x 3 min 5 min
2	**Antigen Retrieval** – Na-citrate buffer (0.1 M, pH 6.0) Microwave (medium high – 700 W) Microwave (medium) Microwave (low) Immerse in cold 0.01 M PBS	 5 min 5 min 5 min
3	Dako PAP marker Wash in 0.01 M PBS	 5 min
4	Block with milk (10% w/w in PBS) Serum blocking solution (10% non-immune serum)	20 min 20 min
5	1^0 **AB** diluted in 0.01 M PBS *__Contr:__* Replace with buffer / non-immune serum Incubate overnight (o/n) at 4^0C in moist chamber	
6	Wash in 0.01 M PBS	3 x 5 min
7	Biotinylated 2^0 AB Wash in 0.01 M PBS	20 min 3 x 5 min
8	Streptavidin-peroxidase conjugate Wash in 0.01 M PBS	20 min 3 x 5 min
9	DAB chromogen Wash in distilled water	10 min 5 min
10	Counterstain in Mayer's haematoxylin Wash in tap water	2 min 5 min
11	Dehydrate tissue through: 70 % EtOH 90 % EtOH 100 % EtOH into Xylene Mount in Entellen (Merck)	3 min each

Table 3.2: Procedure for double-labelling immunohistochemical localization

STEP	TREATMENT	TIME
1		
	Dewax and Hydrate sections	30 min
	Xylene	2 x 3 min
	100 % EtOH	2 x 10 min
	100 % MeOH + 3 % H_2O_2	2 x 3 min
	90 % EtOH	2 x 3 min
	70 % EtOH	5 min
	dH_2O	
2	**Antigen Retrieval** – Na-citrate buffer (0.1 M, pH 6.0)	
	Microwave (medium high – 700 W)	5 min
	Microwave (medium)	5 min
	Microwave (low)	5 min
	Immerse in cold PBS	
3	Dako PAP marker	
	Wash in 0.01M PBS	5 min
4	Block with milk (10% w/w in PBS)	20 min
	Serum blocking solution (10% non-immune serum)	20 min
5	**1^0 PRIMARY ANTIBODY 1**	***Contr:*** Replace with buffer / non-immune serum
	Incubate overnight (o/n) at 4^0C in moist chamber	
6	Wash in 0.01 M PBS	3 x 2 min
7	Biotinylated 2^0 AB	20 min
	Wash in 0.01 M PBS	3 x 2 min
8	Streptavidin-alkaline phosphatase	10 min
	Wash in 0.01 M PBS	3 x 2 min
9	Substrate chromogen for Alkaline phosphatase	10 min
	Wash in distilled water	5 min
10	Double staining enhancer	30 min
	Rinse in distilled water	
	Wash in 0.01 M PBS	3 x 2 min
11	Blocking Serum	20 min
12	**1^0 PRIMARY ANTIBODY 2**	***Contr:*** Replace with buffer / non-immune serum
	Incubate overnight (o/n) at 4^0C in moist chamber	
13	Wash in 0.01 M PBS	3 x 2 min
14	Biotinylated 2^0 AB	20 min
	Wash in 0.01 M PBS	3 x 2 min
15	Streptavidin-peroxidase	10 min
	Wash in 0.01 M PBS	3 x 2 min
16	Substrate chromogen	10 min
	Wash in distilled water	5 min
17	Counterstain in Mayers Haematoxylin	30 sec
	Wash in tap water	5 min
18	Add CLEARMOUNT and heat at 60 ^{0}C	15 - 30 min

CHAPTER FOUR: RESULTS

4.1 Single-Immunolocalization of TK, Kinin B1 and B2 receptors, TGF-β1 and TGF-β receptors II and III in Control (non-cancerous renal tissue) and Renal Carcinoma

TK, Kinin B1 and B2 Receptors, TGF-β1 and TGF-β2 receptors II and III were localized using the peroxidase-antiperoxidase method.

4.1.1 Localization of TK

In the non-cancerous kidney tissue, the labelling experiment localized TK in the distal tubule cells with minimal labelling in proximal tubule cells. No labelling was found in the glomerular mesangial cells or parietal epithelial cells of Bowman's capsule (Figure 4.1).

In the cancerous kidney, there was intense labelling on the distal tubular cells and lesser labelling of the proximal tubular cells. The glomerular mesangial and parietal epithelial cells of Bowman's capsule showed no positive labelling for TK (Figure 4.2).

4.1.2 Localization of Kinin B1 Receptor

In the non-cancerous kidney tissue, labelling for kinin B1 receptor, was minimal in the distal and proximal tubular cells and was not located in the glomerular mesangial cells and parietal epithelial cells of Bowman's capsule. There appears to be marginally higher labelling for kinin B1 receptors in the distal tubular cells compared to the proximal tubular cells as evidenced by the darker staining of the distal tubular cells compared to the proximal tubular cells (Figure 4.3).

In the cancerous kidney tissue, kinin B1 receptors were localized in the distal tubular and proximal tubular cells. The distal tubular cells labelled more intensely for kinin B1 receptors than the proximal tubular cells as evidenced by darker (intense) labelling. In comparison to the non-cancerous kidney tissue, the cancerous kidney tissue shows higher labelling intensity in proximal and distal tubular cells and the presence of kinin B1 receptors in the some glomerular mesangial cells and the parietal epithelial cells of Bowman's capsule (Figure 4.4).

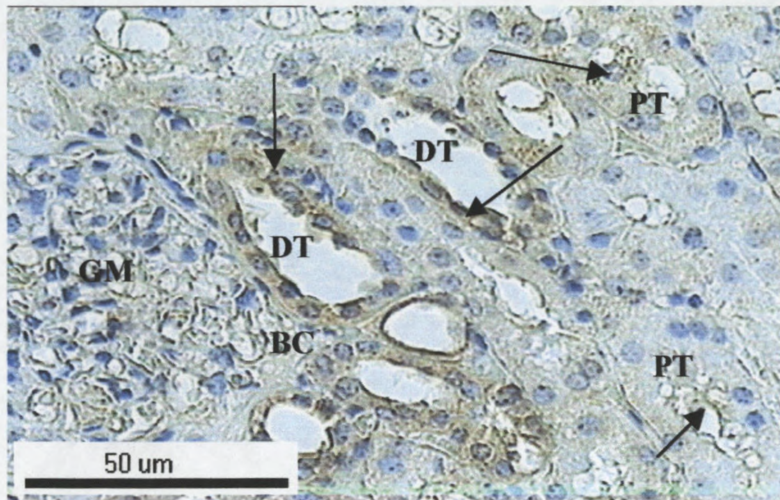

Fig 4.3 Non-cancerous Kidney: photomicrograph (PAP) showing minimal labelling for kinin receptor B1 (arrow) in the distal tubular (DT) and proximal tubular cells (PT). No label was present in the glomerular mesangial cells (GM) and parietal epithelial cells of Bowman's capsule (BC)

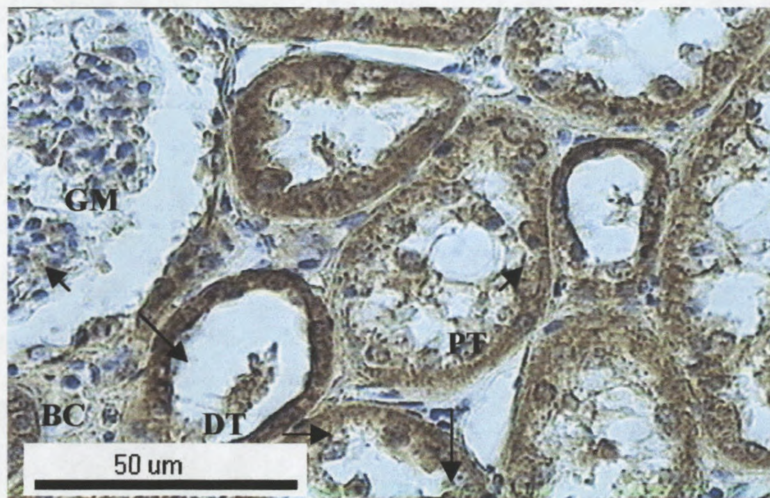

Fig 4.4 Kidney Cancer: photomicrograph (PAP) showing intense positive labelling for kinin receptor B1 (arrow) within the distal tubular (DT) and proximal tubular cells (PT), glomerular mesangial cells (GM) and parietal epithelial cells of Bowman's capsule (BC)

4.1.3 Localization of Kinin B2 Receptor

In the non-cancerous kidney tissue, kinin B2 receptors were present in the cells of the distal tubular and proximal tubular cells. The distal tubular cells stained more strongly for kinin B2 receptors than those of the proximal tubular cells. There is also low inconsistent labelling for the kinin B2 receptors in some glomerular mesangial cells and the parietal epithelial cells of Bowman's capsule (Figure 4.5).

In the cancerous kidney tissue, the distal tubular and proximal tubular cells labelled intensely for the kinin B2 receptor. In comparison to the normal kidney, there was an increase in labelling intensity within the distal tubular and proximal tubular cells. The glomerular mesangial cells and the parietal epithelial cells of Bowman's capsule showed more staining and an inconsistent labelling pattern when compared to the non-cancerous kidney tissue (Figure 4.6).

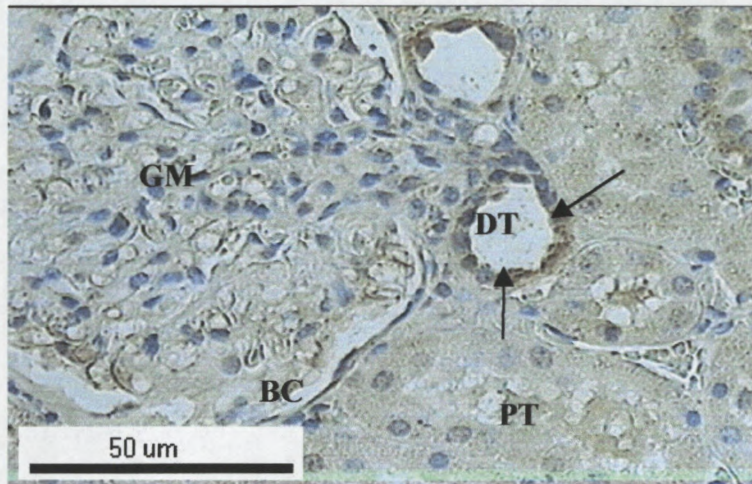

Fig 4.5 Non-cancerous Kidney: photomicrograph (PAP) showing labelling for kinin receptor B2 (arrow) in the distal tubular cells (DT) and no labelling of the proximal tubular cells (PT), parietal epithelial cells of Bowman's capsule (BC) and glomerular mesangial cells (GM)

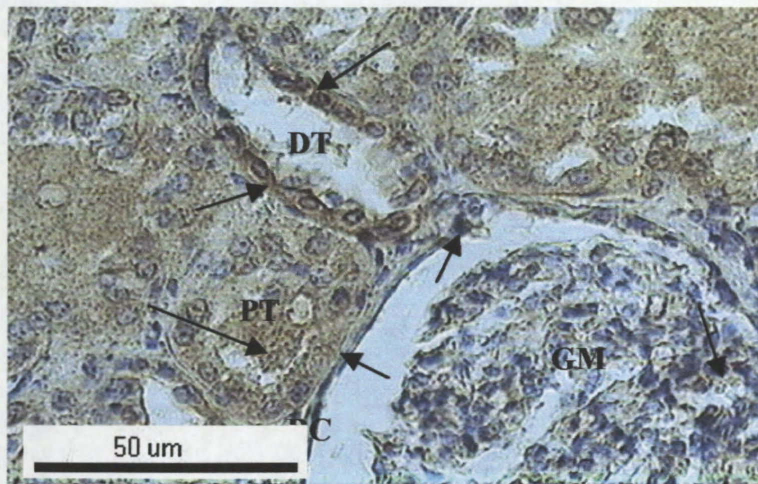

Fig 4.6 Kidney Cancer: photomicrograph (peroxidase-antiperoxidase) showing intense positive staining for kinin receptor B2 (arrow) in the distal tubular (DT) and proximal tubular cells (PT), some glomerular mesangial cells (GM) and parietal epithelial cells of Bowman's capsule (BC)

4.1.4 Localisation of TGF-β1

In the non-cancerous kidney tissue, TGF-β1 was localized in the distal tubular cells and the proximal tubular cells as shown by the dark brown staining (Fig 4.4) of these cells. The distal tubular cells labelled more intensely than the proximal tubular cells as shown by the variability in the staining of the two cells. TGF-β1 was also present in some glomerular mesangial cells and the parietal epithelial cells of Bowman's capsule (Figure 4.7).

In the cancerous kidney tissue the TGF-β1 was observed in the distal tubular and proximal tubular cells, with darker labelling seen in the distal tubular cells. Inconsistent labelling was noted in some glomerular mesangial cells and the parietal epithelial cells of Bowman's capsule. In comparison to the non-cancerous kidney, there is higher labelling intensity in the distal tubular and proximal tubular cells, the mesangial cells and the parietal epithelial cells of Bowman's capsule (Figure 4.8).

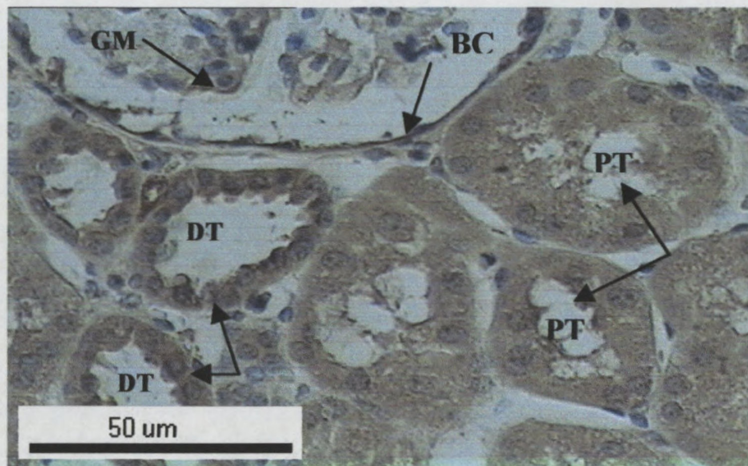

Fig 4.7 Non-cancerous Kidney: photomicrograph (PAP) showing positive labelling for TGF-β1 (arrow) in the distal tubular (DT) and proximal tubular cells (PT), parietal epithelial cells of Bowman's capsule (BC) and some glomerular mesangial cells (GM)

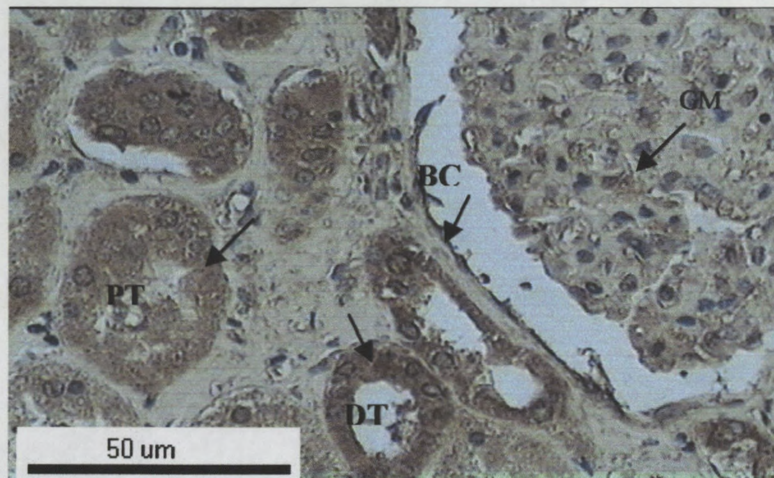

Fig 4.8 Kidney Cancer: photomicrograph (PAP) showing intense labelling for TGF-β1(arrow) in the distal tubular (DT) and proximal tubular cells (PT), some glomerular mesangial cells (GM) and parietal epithelial cells of Bowman's capsule (BC)

4.1.5 Localization of TGF-β Receptor II

In the non-cancerous kidney, both the distal and proximal tubular cells labelled positively for TGF-β Receptor II with a higher labelling intensity present in the distal tubular cells than the proximal tubular cells. The glomerular mesangial cells and the parietal epithelial cells labelled negative (Figure 4.9).

The cancerous kidney tissue showed the same location of TGF-β Receptor II but the distal tubular cells showed an increase in labelling intensity as compared to non-cancerous kidney tissue. No change was seen in the labelling of the proximal tubular cells. As seen with non-cancerous kidney tissue no receptors were observed in the glomerular mesangial cells and the parietal epithelial cells of Bowman's capsule (Figure 4.10).

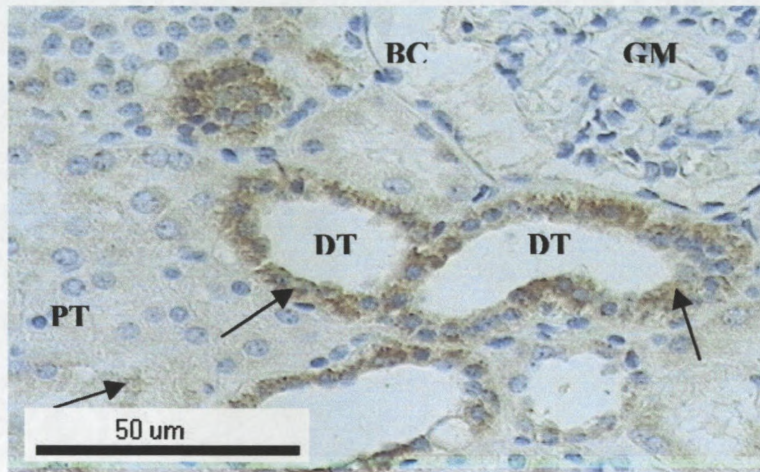

Fig 4.9 Non-cancerous Kidney: photomicrograph (PAP) showing intense positive staining for TGF-β receptor II (arrow) in the distal tubular cells (DT) and minimal staining of the proximal tubular cells (PT). No labelling of parietal epithelial cells of Bowman's capsule (BC) and glomerular mesangial cells (GM)

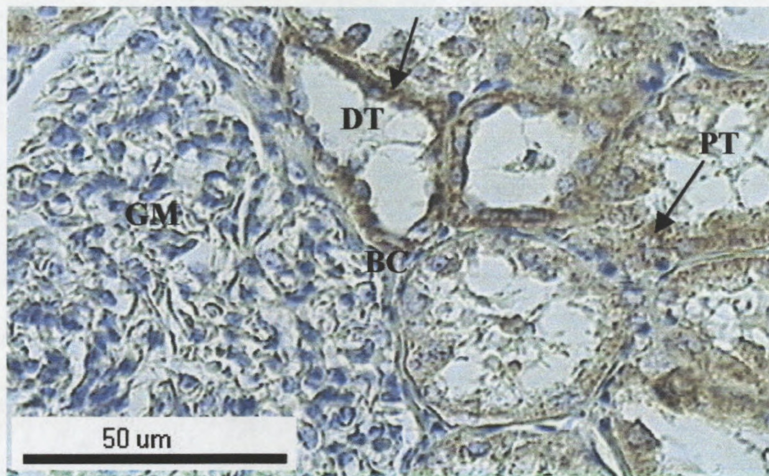

Fig 4.10 Kidney Cancer: photomicrograph (PAP) showing positive label for TGF-β receptor II (arrow) in the distal tubular (DT) and proximal tubular cells. No labelling of the glomerular mesangial cells (GM) and parietal epithelial cells of Bowman's capsule (BC)

4.1.6 Localization of TGF-β Receptor III

In the non-cancerous kidney tissue, low labelling of the TGF-β Receptor III was observed in the distal tubular cells. No TGF-β Receptor III were observed in the proximal tubular cells, glomerular mesangial cells and the parietal epithelial cells of Bowman's capsule (Figure 4.11).

In the cancerous kidney tissue TGF-β Receptor III was observed in both the distal tubular and proximal tubular cells as both areas labelled intensely for Receptor III, with almost no variability between distal and proximal tubular cells (Figure 4.12). In comparison to the non-cancerous kidney tissue, there is a marked increase in label intensity in both distal and proximal tubular cells.

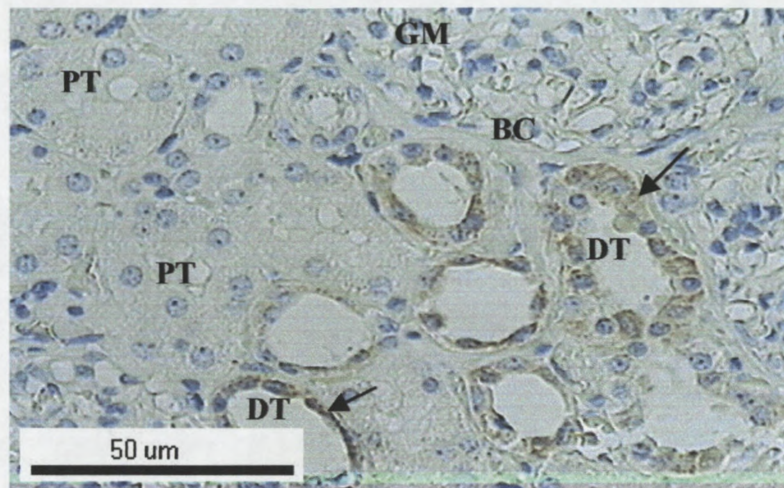

Fig 4.11 Non-cancerous Kidney: photomicrograph (PAP) showing label for TGF-β receptor III (arrow) in the distal tubular cells (DT) and no labelling of the proximal tubular cells (PT), parietal epithelial cells of Bowman's capsule (BC) and glomerular mesangial cells (GM)

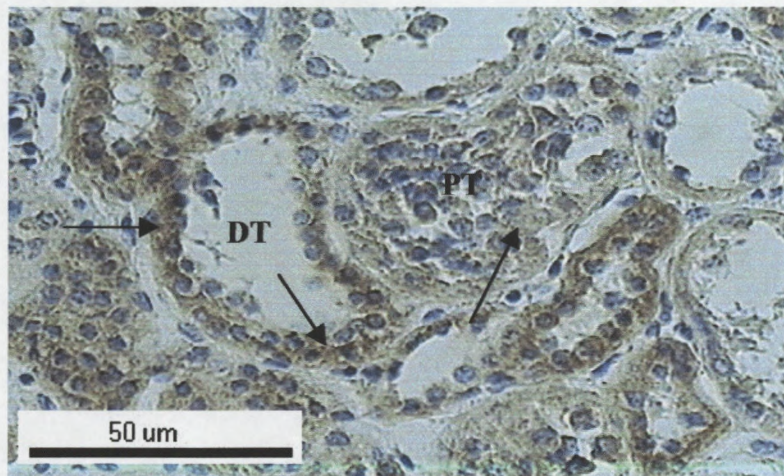

Fig 4.12 Kidney Cancer: photomicrograph (peroxidase-antiperoxidase) showing intense positive staining for TGF-β receptor III (arrow) in the distal tubular (DT) and proximal tubular cells (PT)

4.2 Double-Immunolabelling for TK and TGF-β1 in Control (non-cancerous renal tissue) and Renal Carcinoma

4.2.1 Method Control

Examination of the renal tissue showed typical glomeruli and tubules (Figure 4.13). There was no labelling of the glomerulus and tubules.

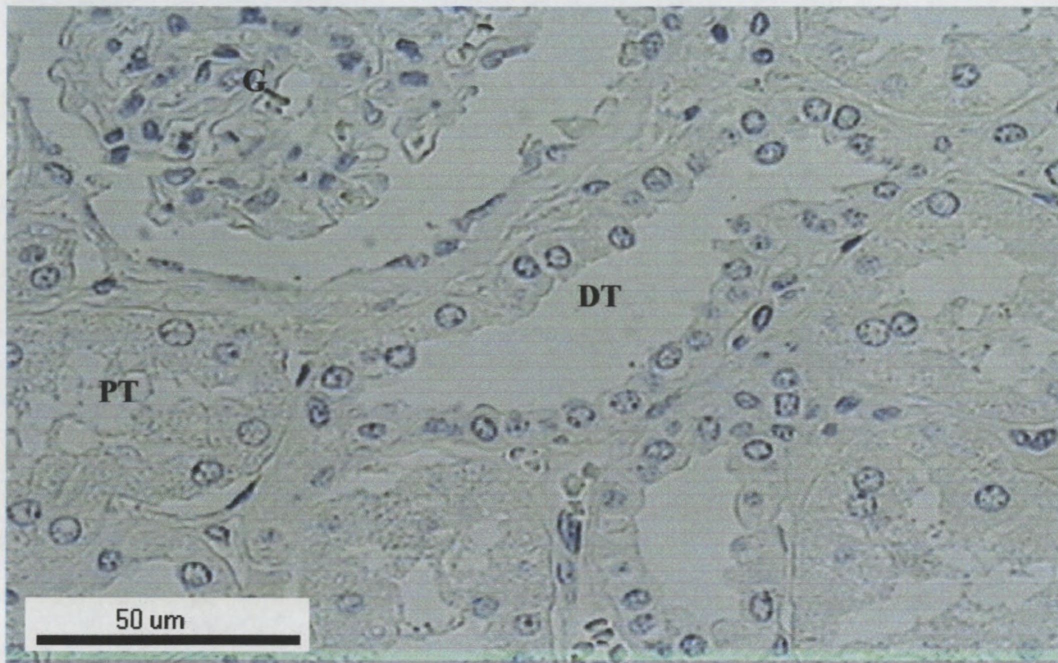

Fig 4.13 Method Control: photomicrograph of renal tissue showing the glomerulus (G), distal tubular cells (DT) and proximal tubular cells (PT)

4.2.2 Co-localization of TK and TGF-β1

In the non-cancerous kidney tissue both TK (red) and TGF-β1 (purple) were present. TK was present in high concentration in both distal and proximal tubular cells. Labelling for TGF-β1 was present within the distal tubular cells and the parietal epithelial cells of Bowman's capsule, at a low labelling intensity. There was also low labelling for TK in the glomerular mesangial cells and the parietal epithelial cells of Bowman's capsule. There was no labelling for TGF-β in the glomerular mesangial cells (Figure 4.14).

In the cancerous kidney tissue, both TGF-β1 and TK was found in higher concentration in the distal tubular and proximal tubular cells, but the labelling intensity for TK was higher than that for TGF-β1 in both the distal tubular and proximal tubular cells. Low inconsistent labelling for both TK and TGF-β1 was observed in the glomerular mesangial and parietal epithelial cells of Bowman's capsule. In comparison to non-cancerous kidney tissue, there was an increase in the labelling intensity for TK and TGF-β1 (Figure 4.15).

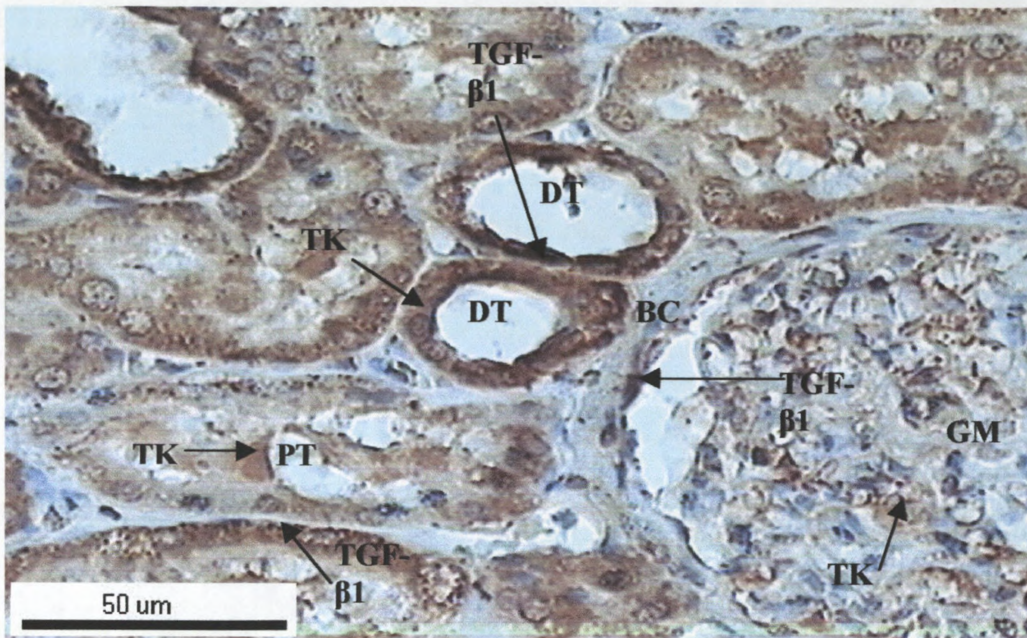

Fig 4.14 Non-cancerous Kidney: photomicrograph showing positive labelling for TK (red) in the distal tubular (DT) and proximal tubular cells (PT), and some glomerular mesangial cells (GM). Minimal labelling for TGF-β1 (purple) in the distal tubular cells, proximal tubular cells and parietal epithelial cells of Bowman's capsule (BC). There was no labelling for TK in the parietal epithelial cells of Bowman's capsule and no labelling for TGF-β1 in the glomerular mesangial cells.

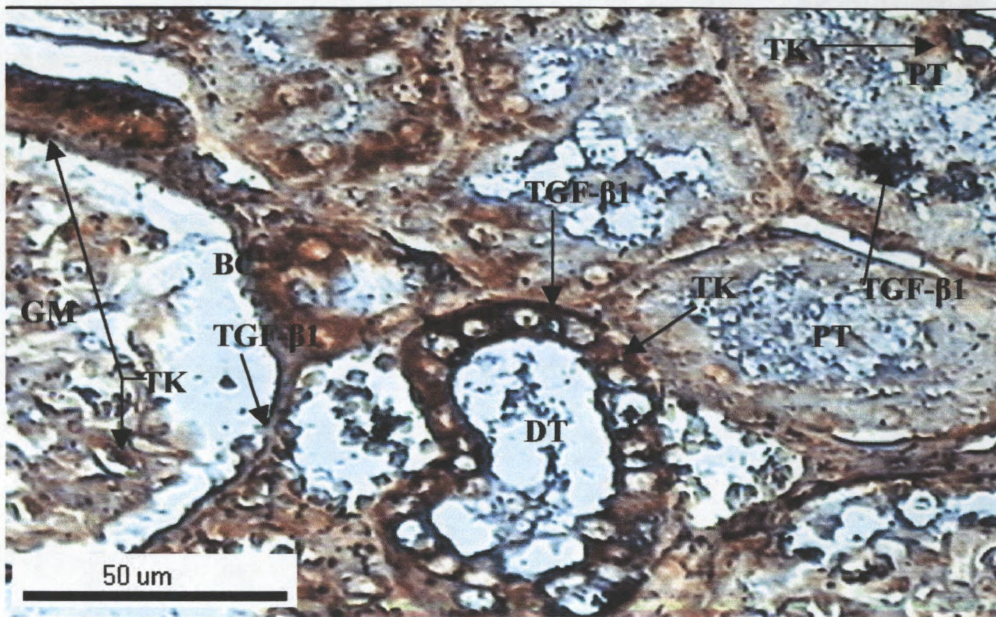

Fig 4.15 Kidney Cancer: photomicrograph showing positive staining for TK in the distal tubular (DT) and proximal tubular cells (PT), some glomerular mesangial cells (GM) and parietal epithelial cells of Bowman's capsule (BC). Minimal labelling for TGF-β1 in the distal and proximal tubular cells, and parietal epithelial cells of Bowman's capsule. No labelling of the glomerular mesangial cells.

CHAPTER 5: DISCUSSION

The present immunohistochemical study focussed on the localization of TK, kinin B1 and B2 receptors, TGF-β1, TGF-β receptors II and III and the co-localization of TK and TGF-β1 in non-cancerous and cancerous human kidney.

The Tissue Kallikrein-Kinin System

Although the primary function of the kidney is regulation of the volume and ionic composition of body fluids, it is also an endocrine organ with an important role in the regulation of blood pressure. Some renal hormones have both vasoconstrictor and antinatriuretic effects (vasopressor systems) while others have vasodilator and natriuretic effects (vasodepressor systems). A characteristic of these systems is that they act not only on distant organs but also in the kidney itself. For example, the renin-angiotensin, prostaglandin and kallikrein-kinin systems, whose activation and actions appear to be interrelated, may participate in the control of blood pressure not only by altering the tone of extrarenal blood vessels, but also by directly regulating intrarenal sodium and water excretion, perhaps by regulating renal blood flow distribution. Studies have shown that the renal kallikrein-kinin system is involved in the regulation of renal blood flow, water and sodium excretion and the release of renin (Chen *et al.,* 1995).

A number of studies have shown that the release of renal tissue kallikrein is associated with several factors such as high sodium or potassium intake, antidiuretic hormone effect and neural control (Lauar *et al.*, 1982; Lauar and Bhoola, 1986; Vio and Figueroa, 1987). Being a crucial aspect to the understanding of its function, the localization of TK has been an issue for decades. Cellular localization of TK in the human nephron proved to be difficult. Initial studies on the human kidney failed to localize TK however, in subsequent studies immunolabelled results confirmed the presence of tissue kallikrein in the normal renal cortex, within the connecting tubular cells of the DT but not in the PT (Figueroa *et*

al., 1988; Ramsaroop *et al.*, 1997; Vio and Figueroa, 1988). However, the presence of TK in the PT was reported in previous studies as reabsorption droplets (Scicli and Carretero, 1986). In the human kidney, TK mRNA has been localized in the DT and collecting ducts, renal blood vessels, juxtaglomerular cells and glomeruli (Cummings *et al.*, 1994; Chen *et al.*, 1995).

Immunoreactive low molecular weight kininogen has also been localized in the DT and collecting ducts of the human kidney (Figueroa *et al.*, 1988). Anatomical co-localization of kallikrein and the substrate kininogen in the kidney suggests an intrinsic renal kallikrein-kinin system, in which intrarenal production of kinins may increase local renal blood flow, glomerular filtration, electrogenic ion transport as well as maintaining renal homeostasis. All these potent effects of kinins are presumably mediated via one or more specific receptors that have been classified into two types named B1 and B2 receptors.

Whereas the B2 receptor is constitutive and activated by the parent molecules, the B1 receptor is generally underexpressed in normal tissues. The induction and increased expression of the B1 receptors occurs during physiological insults such as trauma, tissue damage or inflammation. B1 receptors are also upregulated by cytokines, endotoxins and growth factors. In an attempt to determine the contribution of kinin B1 receptors to kinin action in the kidney, Marin-Castano *et al.* (1998) investigated its distribution under physiological conditions and no B1 receptor mRNA could be detected under physiological conditions. Studies done by Bhoola *et al.* (2001) confirmed the absence of kinin B1 receptors in normal renal cells.

Kinin B2 receptors are constitutively expressed in most normal tissues and are thought to play a role in nonpathological conditions as well as in acute inflammatory and pain responses. B2 receptor distribution along the nephron has been extensively studied since this receptor mediates the larger part of the kinin responses under physiological conditions. Immunohistochemical studies by Ramsaroop *et al.* (1997) localized immunoreactive kinin B2 receptors in the GM, PT, DT and BC. The mRNA for kinin B2 receptor was localized by Bhoola *et al.* (2001) in both the cortex and medulla of the

kidneys, mesangium, epithelial cells of Bowman's capsule, PT and DT, endothelial cells of vessels, thick and thin loops of Henle, collecting ducts and juxtaglomerular cells.

Localization of TK

Non-cancerous kidney: In this study, the non-cancerous kidney cells showed minimal labeling for TK in the proximal tubular cells. This finding is in contrast to previous literature on the localization of TK in which; human renal TK is restricted to the connecting tubule (CNT). The junction between a CNT and an unstained cortical collecting duct (CCD) has seen in Fig 4.1 (6, 9). TK has been reported to be present in the cells of the connecting segment of the distal tubule (Figueroa *et al.*, 1988; Ramsaroop *et al.*, 1997). Within the cell, TK is concentrated mainly along the luminal membrane and, in response to specific stimuli, is secreted into the tubular lumen. Kininogen has also been localized to tubular cells of the distal nephron (Cummings *et al.*, 1994). Fig 4.1 (7a, 7b, 8) shows the close relationship between kallikrein (brown) and kininogen (purplish), with both cells coexisting in the same tubule but are sited in separate cells.

In accordance with previous studies on the localization of TK, the results of the current study confirmed that the dominant site of immunoreactivity for TK is the DT. A weak but definite immunoreactivity was observed in PT. This could be attributed to the use of non-cancerous kidney tissue that may have been from a hypertensive or nephritic glomerular disease kidney, instead of normal kidney. The GM cells and the BC showed no positive labelling for TK although a recent study conducted by Ramsaroop *et al.* (1997) reported the presence of TK in the BC.

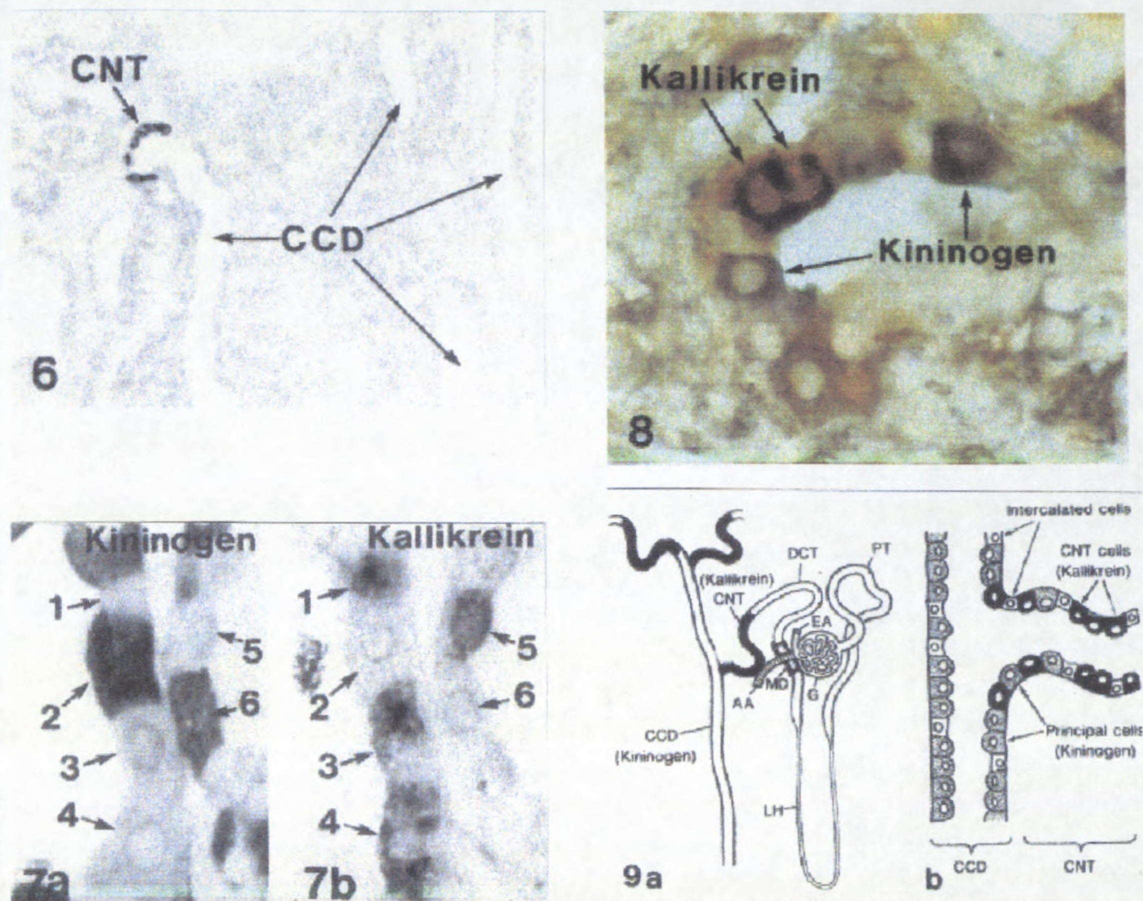

Fig 5.1 Localization of human tissue kallikrein in the normal kidney (Figueroa *et al.*, 1988)

Cancerous kidney: There was intense immunolabeling of TK in the DT. The kallikrein cascade is implicated in tumourigenesis, firstly by induction of kallikrein gene expression as the initial step of the cascade, and then sequentially by the formation of kinins. Renal kallikrein as a part of the kallikrein-kinin system is involved in many disease processes, including inflammation, hypertension, renal disease and cancer. The TK gene in the kidney may not be constitutively expressed in the kidney, but is expressed in response to physiological and pathological stimuli. Gene expression of tissue kallikrein, and the subsequent formation of the vasoactive peptides could stimulate proliferation of tumour cells, and by increasing vascular permeability, enhance metastasis

(Roberts, 1989). Therefore, it is considered that TK might be implicated in the process of tumourigenesis both as a serine protease as well as through the cellular actions of bradykinin (Roberts and Gullick, 1989). Recent studies have also suggested the possibility of an increased expression of tissue kallikrein in cancer, arising from the induction of one or more of the kallikrein genes (Dlamini *et al.*, 1999).

Localization of Kinin B1 Receptor

Non-cancerous kidney: Kidney receptor status showed B1 receptor immunoreactivity in the DT and PT and negative labeling in the GM and BC. The positive expression of B1 receptor could be attributed to the use of a non-cancerous kidney tissue. Under physiological conditions the B1 receptor is not expressed at significant levels, but its expression is often induced during inflammation therefore, the possible inflammation or tissue damage of the kidney section may have resulted in the expression of the B1 receptor.

Cancerous kidney: It is now established that kinin B1 receptors are not constitutively expressed in normal tissue. Evidence suggests that these receptors are induced during inflammation and tumour growth. Kinin B1 receptor showed intense immunoreactivity in the DT and PT, whereas the GM and BC capsule showed some moderately intense labeling for the B1 receptor, unlike the non-cancerous kidney tissue which was negative. The observation of increased labeling for the B1 receptor suggests a site for kinin activity and a possible mitogenic role for kinins in cancerous cells.

Localization of Kinin B2 Receptor

The distribution of the B2 receptor in the kidney has been widely studied in several species using different techniques. The cellular localization of B2 receptor mRNA in the human kidney was reported (Song *et al.*, 1996). Taken together, these studies have allowed a precise localization of the B2 receptor along the nephron.

Non-cancerous kidney: Kinin B2 receptor immunolabelling was visualized in the DT and PT, GM and BC as reported by previous studies. Kinin B2 receptor did not label intensely in the PT, GM and BC. This could be accredited to the use of a non-cancerous kidney tissue that may have been inflamed or undergone tissue damage, in which case the kinin B1 receptor would be induced.

Cancerous kidney: There are limited studies on the status of the kinin B2 receptor in pathological studies. Kinin B2 receptors are constitutively expressed and are responsible for the physiological actions of kinins (Naicker *et al.*, 1999). The current study showed intense expression of the B2 receptor in the DT and PT, GM and BC in the cancerous kidney, in contrast to the non-cancerous kidney.

TGF-β1 system

TGF-β1 has been known to play a role both in the process of normal development and in the pathogenesis of a wide variety of disease processes in the kidney. The production of TGF-β1 and the expression of TGF-β receptors are typical features of renal cells. TGF-β1 exerts its effects through binding to these specific cell-surface receptors that act co-operatively. Three types of TGF-β receptors have been identified: type I (TβR-I), type II (TβR-II) and type III (TβR-III). TβR-III is a proteoglycan. It has a short intracellular domain and contains no apparent signalling motif. The biologic function of TβR-III may involve presentation of TGF-β to target cells and does not directly participate in signal tranduction.

TGF-β1 regulates diverse cellular functions via a heteromeric signaling complex of transmembrane serine/threonine kinase receptors. The TGF-β1 receptors are a complex group of receptors. Virtually all cells have TGF-β1 receptors that control a variety of functions in cells from essentially every lineage. Glomerular endothelial, mesangial and epithelial cells all express TGF-β1 receptors and respond to TGF-β1 (Shankland *et al.*,

1996). Both TβR-I and TβR-II are required for the expression of TGF-β1 biologic effects. Consequently, cells with a loss of expression or a mutational inactivation in either type of the receptor will become TGF-β insensitive.

The TGF-β system has a wide variety of biological effects and plays important although poorly understood roles in the normal renal structure and glomerular and tubular function. TGF-β1 has been best investigated among all three isoforms, and the expression of TGF-β1 in the normal kidney suggests its involvement in physiologic renal function. TGF-β1 is the most highly expressed, being present mainly in tubular epithelial cells and to a lesser extent in the glomerulus (MacKay *et al.*, 1990). In the adult kidney, TGF-β1 is involved in the day-to day normal maintenance of renal function. TGF-β1 modulates renal secretion of parathyroid hormone related protein by renal epithelial cells, decreases tubular phosphate reabsorption and is a potent stimulator of renin secretion by renal juxtaglomerular cells (Law *et al.*, 1993). TGF-β1 also plays a crucial role in regulating cell proliferation and extracellular matrix production. In the kidney, TGF-β1 promotes tubuloepithelial cell hypertrophy and regulates the glomerular production of almost every known molecule of the extracellular matrix. TGF-β1 also blocks the destruction of newly synthesized extracellular matrix by up-regulating the synthesis of protease inhibitors, and by down-regulating the synthesis of matrix-degrading proteases (Sharma and Ziyadeh, 1994). The ability of TGF-β1 to enhance matrix synthesis underlies its important role in healing. Renal TGF-β1 serves to promote tissue regeneration. TGF-β1 prevents over tubularization of the renal ductules and acts to shape the correct architecture of the normal mature kidney (Rogers *et al.*, 1993) In injured cells, TGF-β1 has been localized predominantly in the regenerating tubules. As a classical antimitogen, its potent antiproliferative activity is another characteristic effect of TGF-β1 on renal cells such as proximal tubular cells, glomerular epithelial cells and glomerular endothelial cells (Anderson *et al.*, 1993; Sharma and Ziyadeh, 1994). However, in certain mesenchymally derived cells (e.g. mesangial cells, smooth muscle cells, osteoblasts and fibroblasts), TGF-β1 acts as a bifunctional regulator of growth; it may either stimulate or inhibit proliferation depending on cell culture conditions, TGF-β1 concentration, or secondary activation of counterregulatory cytokine systems (Battegay *et al.*, 1990).

Recent studies indicate that the TGF-β family binds to type I and type II serine/threonine kinase receptors, which initiate intracellular signals through the activation of SMAD proteins. Receptor regulated SMADs are phosphorylated by the receptors and form oligomeric complex with common partner SMADs, The oligomeric SMADs complexes then translocate into the nucleus, where they regulate the transcription of target genes by direct binding to DNA, interactions with various DNA-binding proteins and recruitment of transcriptional co-activators or co-repressor. In the case of cell growth inhibition by TGF-beta1 the activated SMADs (Smad 2/3/4 complex) translocate into the nucleus where they promote the activities of CDK inhibitors (p21 and p15), which inhibit the activites of CDK4/ cyclin D and CDK2/ cyclin E complexes. For these phenomena the protein product of the retinoblastoma gene pRB, which has properties of a cell cycle regulatory factor, are maintained in an underphosphorylated state. As a result, cells in the G1 phase of the cell cycle are not able to proceed to the S-phase and cell proliferation is inhibited (Heldin *et al.*, 1997; Massague, 1998; Miyazono *et al.*, 2000).

Localization of TGF-β1

Non-cancerous kidney: Immunohistochemical studies on the normal kidneys have localized TGF-β1 to glomeruli mesangial, glomerular epithelial cells, juxtaglomerular and tubular epithelial cells (MacKay *et al.*, 1990). More recent reports have detected TGF-β1 mRNA in the glomeruli and all segments of the renal tubules, predominantly the DT. Cells in all these nephron segments constitutively express TGF-β1 (Ando *et al.*, 1998). In harmony with these findings, the present study immunolocalized TGF-β1 in the DT and PT with labelling predominate in the DT. TGF-β1 labelling was also present in the GM and BC.

Cancerous kidney: The resistance to TGF-β1's inhibitory effect on cell proliferation has provided cancer cells with an added growth advantage over their benign counterparts.

TGF-β1 action is a ubiquitous biological phenomenon in that, under physiological conditions, it provides a mechanism for cellular homeostasis. In cancer cells, uncontrolled growth can result in an escape from this homeostatic constraint. In contrast to the non-cancerous kidney tissue, more intense labelling for TGF-β1 was observed in the DT and PT, GM and BC. These results confirm previous reports of increased TGF-β1 in cancer (Ramp *et al.*, 1997). Thus the increased expression of TGF-β1 in transformed and non-transformed cells is indicative of its regulatory effects in cancer and the transformation of cells is due to the lack of responsiveness to TGF-β1.

Localization of TGF-β1 Receptor II

TGF-β1 regulates diverse cellular functions via a heteromeric signaling complex of transmembrane serine/threonine kinase receptors. The TGF-β1 receptors are a complex group of receptors. Virtually all cells have TGF-β1 receptors that control a variety of functions in cells from essentially every lineage. Glomerular endothelial, mesangial and epithelial cells all express TGF-β1 receptors and respond to TGF-β1 (Shankland *et al.*, 1996). Both TβR-I and TβR-II are required for the expression of TGF-β1 biologic effects. Consequently, cells with a loss of expression or a mutational inactivation in either type of the receptor will become TGF-β insensitive.

Non-cancerous kidney: Receptor II mRNA and Receptor II expression has been found in glomeruli, arterioles and tubular segments, predominantly in the distal nephron (Ando *et al.*, 1998; Shankland *et al.*, 1996). In the current study receptor II was intensely immunolabeled in the DT and with much lower intensity in the PT. The GM and BC showed no labeling. The localization of receptor II correlates with that of TGF-β1 suggesting that there is induction of growth regulation by TGF-β1 through the process of signal transduction.

Cancerous kidney: The action of TGF-β1 requires the presence of functionally active type II receptors. It has been previously demonstrated that cancer cells are insensitive to the inhibitory effect of TGF-β1 due to the absence of expression of type II receptor.

Immunohistochemical analysis has demonstrated persistent expression TGF-β1 type II receptors in cancer (Ramp *et al.*, 1997). The increased expression of receptor II in the DT and constant labelling of the PT and the co-expression of TGF-β1 and receptor II in this site, suggests a site for the regulation of proliferation of the cancer cells.

Localization of TGF-β1 Receptor III

TGF-β1 type III receptor is a molecule with proteoglycan structure. Although receptor III is not involved in the signal transduction of TGF-β1, there is evidence that the type III receptor increases binding of TGF-β1 to the type II receptor (Wang *et al.*, 1991).

Non-cancerous kidney: Ando *et al.* (1998) recently demonstrated that receptor III mRNA is expressed in glomeruli and tubules. In the present study, receptor III was localized to the DT. The presence of receptor III at this predominant site, further suggests its role in ligand presentation of TGF-β1 to receptor II as both TGF-β1 and receptor II have also been localized to this site.

Cancerous kidney: The biologic function of the type III receptor involves the presentation of TGF-β1 to target cells and receptor II. Thus, the intense expression of receptor III in the DT and PT in the present study in conjunction with the localization of TGF-β1 and receptor II at these sites further suggests its role in ligand presentation, leading to the regulation of cancer proliferation in the cancerous kidney.

Kallikrein-Kinin and TGF-β1 systems

Non-cancerous kidney: In harmony with previous studies, this study independently localized TK and TGF-β1 to the DT and PT cells using single-immunolabeling (Table 5.1). These results were further confirmed by the co-localization of TK and TGF-β1 in these cells using double-immunolabeling. The presence of TK and TGF-β1 in the non-cancerous kidney further suggests their roles in the normal functioning of the human kidney.

Cancerous kidney: TK and TGF-β1 were further localized to the DT and PT in the cancerous kidney, using double-immunolabeling, confirming results obtained during single-immunolabeling of the cancerous kidney tissue (Table 5.1). The presence of TK and TGF-β1 in these cells suggest that this is the predominant site of expression and activity of these modulates and suggests their mitogenic and antiproliferative effects in pathological states, respectively. The co-localization of TK and TGF-β1 also proposes a possible relationship between the two.

All components of the kallikrein cascade have been identified in acute inflammatory reactions (Rahman *et al.*, 1994). A molecular response to infection, tissue injury and proliferation of tumour cells is the secretion of chemotactic molecules that attract neutrophils to sites of inflammation. When neutrophils are drawn into loci of infection, into inflamed joints or into tumours, activation of the kallikreins initiates a cascade of molecular events that play a crucial role in the specific inflammatory process. Kinins formed by the enzymatic action of kallikreins are attractive autocoids that mediate oedema and pain, cardinal features of acute inflammation (Bhoola *et al.*, 2001). Kinins initiate their cellular actions through two receptors, kinin B2 and kinin B1. Bradykinin and the related kinin, lys-bradykinin (kallidin), are formed following inflammatory insult or tissue injury. The bradykinin B2 receptor is constitutively expressed by most cells and is thought to play a role in nonpathological conditions as well as in acute inflammatory and pain responses. By contrast, the bradykinin B1 receptor is highly inducible and may be expressed in chronic inflammation (Newton *et al.*, 2002).

During tumourigenesis, gene induction of TK, and the subsequently formed kinins stimulate proliferation of tumour cells by their mitogenic actions and additionally promote the invasion of normal tissue as well as diapedesis. Kinins are able to increase local blood flow and permeability, thus providing an environment for the diapedesis and metastatic migration of cancer cells. The bradykinin B1 receptor is expressed in human tumours, which suggests a potential role for bradykinin in inducing pathologic signal transduction in cancer growth and progression, nitric oxide production and vascular permeability enhancement in tumours (Wu *et al.*, 2002).

The kallikrein-kinin system has been shown to activate angiogenesis in an *in vivo* mouse model (Emanueli *et al.*, 2001). Previous studies suggest that kinins formed by kallikrein may promote the angiogenic cascade (Bhoola *et al.*, 2001). TK and kinins in the sequential steps that form the angiogenic cascade lead to the formation of new blood vessels. Furthermore, tumour growth and metastasis depends on the process of angiogenesis. Serine proteases are involved in tumour progression, such as invasion, proliferation and tumour metastasis (Carroll and Binder, 1999). Strong experimental evidence supports a link between kallikreins and endocrine malignancies. More recently, kallikreins and kinins have been implicated in the carcinogenic process (Diamandis *et al.*, 2000). Given the co-expression of many kallikreins in cancer, it is reasonable to postulate that kallikreins are involved in a cascade enzymatic pathway that plays a role in cancer progression (Yousef and Diamandis, 2002).

TGF-β1 is a key mediator of the immune and inflammatory responses including controlling the differentiation of T cells and deactivation of macrophages. TGF-β decreases the proliferation and cytokine synthesis of T and B cells (Del Giudice and Crow, 1993). TGF-β1 deactivates macrophages and has been shown to decrease nitric oxide (NO) production (Ding *et al.*, 1990), to decrease pro-inflammatory cytokines (Espevik *et al.*, 1987), increase the synthesis of matrix proteins and decrease the synthesis of proteases (Sharma and Ziyadeh, 1994). The balance of anti-inflammatory and pro-fibrotic effects is probably determined by the stage of inflammation and the predominant cell types involved. Early on during the evolution of the inflammatory response TGF-β1

acts as a negative controller, while as injury progresses and severity of inflammatory cell infiltrate declines its effects promote scarring.

The most prominent characteristics of malignant neoplasms (carcinoma) are that these cells have a decrease in or lack of control mechanisms of growth (uncontrolled growth), invasion to local tissues (invasion) and the abilities to spread from their sites of origin to lodge and grow at other locations in the body (metastasis). Benign tumour cells also have decreased control mechanisms of growth but these cells do not invade or spread. In cancer cells the structure and/or regulation of genes, which regulate cancer cell growth or controls the interactions of cancer cells and normal cells, is destroyed and cancer cells are able to attain growth advantages through a succession of mutational events (Vogelstein *et al.*, 1988). The balance of normal growth regulation in tumours can, however be profoundly disturbed by defects in growth-inhibitory systems.

In particular, TGF-β1 has recently been identified as one of the most potent inhibitory growth factor for epithelial cells, also affecting the proliferation of non-neoplastic and neoplastic epithelial kidney cells (Reddy *et al.*, 1994; Alexandrow and Moses, 1995). TGF-β1 is secreted by many cells that may participate in the host response to tumourigenesis including activated T-cells, B-cells, macrophages and lymphokine-activated killer cells. The overall effect of TGF-β1 on neoplastic cell growth may be determined by the relative contributions of various cell types in the tumour microenvironment and whether local TGF-β1 is latent or activated (Gomella *et al.*, 1989). TGF-β1 may also effect the growth of target cells indirectly by altering the production of and response to other growth factors

Many cancer cells develop an aggressive growth pattern due to their loss of sensitivity to the inhibitory effect of TGF-β1 (Park *et al.*, 1994; Kundu *et al.*, 1998). Since most tissue contain TGF-β1, the widespread distribution of its receptors supports a fundamental role for this factor in the autocrine and paracrine growth regulation of normal and transformed cells (Shimabukuro *et al.*, 2003). Evidence is accumulating to suggest that alterations in the expression of TGF-β receptors may play a critical role. According to the current

understanding, TGF-β1 directly binds to type II receptor, which then heterodimerizes with the type I receptor, thereby activating downstream signal transduction (Wrana *et al.*, 1994). Moreover, type III receptor is thought to be involved in ligand presentation to type II receptor (Moustakas *et al.*, 1993). TβR-I and TβR-II are required for proper manifestation of TGF-β1 action (Chen and Weinberg, 1995). TGF-β1 signal transduction requires both receptors to be present, the loss of either TβRI or TβRII could allow cancer cells to escape the growth inhibitory effect of TGF-β1 and acquire a growth advantage. The ability of cancer cells to become insensitive to the growth inhibitory effects of TGF-β is most commonly due to alterations in the TβR-II gene. This leads to either a truncated or fully absent mRNA transcript, with resultant TGF-β1 insensivity (Engel *et al.*, 1999). In some cancer lines, TβR-II mutational events occur more frequently that any other types of mutation, and TβR-I mutations are relatively rare (Chang *et al.*, 1997). The list of malignant cell types that have been shown to escape TGF-β1 growth suppression and gain malignant potential because of a loss of functional TβRII has become extensive. It includes colon, stomach, head and neck, endometrium, pancreas, breast, prostate, retinoblastoma, T-cell lymphoma, and liver (Inagaki *et al.*, 1993; Park *et al.*, 1994; Myeroff *et al.*, 1995; Kim *et al.*, 1996; Chang *et al.*, 1997). Recently, human RCC (Ramp *et al.*, 1997) and mouse renal carcinoma line, Renca, have been added to the list (Kundu *et al.*, 1998). The central role of TβR-II in growth regulation, coupled with progression to a more aggressive phenotype in cancer cells on its loss, supports the concept that TβR-II is a tumour suppressor gene.

Paradoxically, most cancer cell studied thus far expressed an elevated level of TGF-β1. Experimental results demonstrate that TGF-β1 enhances tumourigenicity in several tumour types. It has been postulated that cancer cells become resistant to the growth inhibitory effect of TGF-β1. Thus, the expression of the growth factor by cancer cells may likely provide these cells with an additional survival advantage because angiogenesis is enhanced and immune response is blunted (Kundu *et al.*, 1998).

TK in tumourigenesis may convert precursor proteases and macromolecules into active molecules. It was recently reported that TK activates in vitro matrix degrading metallo-proteases present in carcinoma cells. The secretion of TGF-β1 as a latent complex necessitates the existence of a regulated activation process, which is most probably mediated through the activities of proteases that preferentially degrade the TGF-β1 pro-segments and thereby release the highly stable, active TGF-β1 dimer (Derynck *et al.*, 2001). Latent TGF-β1 can also be activated by the metallo-proteases MMP-9 and MMP-2 (Yu and Stamenkovic, 2000), which is frequently expressed by malignant cells, especially at sites of tumour cell invasion (Stamenkovic, 2000). The subsequent activation of TGF-β1 results in the production of extracellular matrix and the inhibition of the proliferation of epithelial tumour cells. Fig 5.2 is a diagrammatic representation of the above statement.

Based on the results of this study, it is hypothesized that TK regulates the expression of TGF-β1 and TGF-β1 regulates the proliferation of the cancer cells.

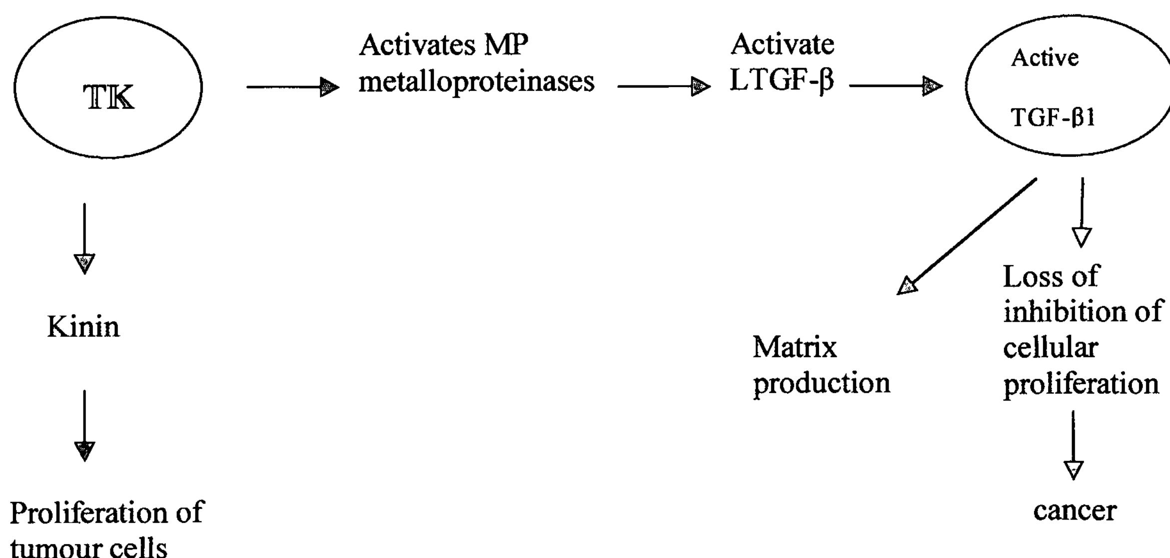

Fig 5.2 Schematic representation of TK and TGF-β1 activity

Table 5.1: Summary of single-immunolabeling results

ANTIBODY	RENAL CELLS	SINGLE – IMMUNOLABELLING	
		NORMAL KIDNEY	CANCER KIDNEY
TK	DT	++++	+++
	PT	++	+
	GM	-	-
	BC	-	-
ANTI-B1	DT	++	+++++
	PT	+	++++
	GM	-	+
	BC	-	+
ANTI-B2	DT	+++	++++
	PT	-	+++
	GM	-	+
	BC	+	+
TGF-β1	DT	++++	+++++
	PT	+++	++++
	GM	+	+
	BC	+	+
TGF-β RII	DT	++++	++++
	PT	+	+
	GM	-	-
	BC	-	-
TGF-β RIII	DT	++	+++
	PT	-	++
	GM	-	-
	BC	-	-

RATING

+++++	very dark labelling
++++	dark labelling
+++	fair labelling
++	minimal labelling
+	inconsistent labelling
-	no labelling

CHAPTER SIX: CONCLUSION

Tissue kallikrein has been implicated in the carcinogenic process and TGF-β1 is a known potent inhibitor in most cells, especially of epithelial lineage (epithelial carcinoma included). TK and TGF-β1 have been previously independently localized in the kidney but no studies have thus far been carried out on the co-localization of TK and TGF-β1 in the kidney. Therefore, the aim of this study was to co-localize TK and TGF-β1 in kidney cancer in order to gain an insight into the biological activity between TK and TGF-β1. The localization of TK confirms the celluar orientation of renal TK in the distal tubular cells of the human kidney. The proximal tubular cells showed minimal labelling assumed to be due to glomerular disease in the non-cancerous renal tissue. The glomerular mesangial cells and parietal epithelial cells of Bowman;s capsule did not show labelling for TK. In the cancerous kidney even though the distal tubular cells showed intense immunostaining, there seemed to be a decrease in the intensity of the labelling. TK expression was confirmed in the parietal epithelial cells of Bowman's capsule, this finding may reflect a site for action of kinins. Kinin B1 receptors were visualised in the distal tubular and proximal tubular cells of the non-cancerous kidney tissue. In the cancerous kidney tissue, induction of the kinin B1 receptor was demonstrated by the intense label of the distal and proximal tubular cells. There was less prominent immunolabel on the glomerular mesangial cells and parietal epithelial cells of Bowman's capsule. Kinin B2 receptor localization was demonstrated on the distal tubular cells and no labelling on the proximal tubular cells, glomerular mesangial cells and parietal epithelial cells of Bowman's capsule. There is an overall increase in the intensity of label for Kinin B2 receptor in the distal tubular cells of the cancerous kidney and presence of labelling for kinin B2 receptor proximal tubular cells, glomerular mesangial cells and parietal epithelial cells of Bowman's capsule. TGF-β1 was identified in both the non-cancerous and cancerous kidney tissue and the sites of visualisation included the distal tubular cells, proximal tubular cells, glomerular mesangial cells and parietal epithelial cells of Bowman's capsule. TGF-β receptor II localization was demonstrated predominantly on the distal tubular cells. The proximal tubular cells, glomerular

mesangial cells and parietal epithelial cells of Bowman's capsule did not show immunolabelling for receptor II. In the cancerous kidney, there was an increase in the intensity of label for receptor II in the distal tubular and proximal tubular cells. This correlates with the increase in TGF-β1 expression in the cancerous kidney tissue. TGF-β receptor III was visualised on the distal tubular cells only. In the cancerous kidney there was positive immunostaining for receptor III in the proximal tubular cells and an increase in the intensity of labelling on the distal tubular cells. The above results were demonstrated by immunostaining utilising the peroxidase-antiperoxidase (PAP) method.

The co-localization of TK and TGF-β1 further confirmed the single-immunolabelling results, with an overall increase in intensity of label for both TK and TGF-β1 in the cancerous kidney tissue. The results of this study showed that TK and TGF-β1 co-localize in the human kidney and hence we hypothesized that TGF-β1 may regulate the expression of tissue kallikrein, and therefore because of its anti-mitogenic property the proliferative and mitogenic actions of kinins on cancer cell.

CHAPTER SEVEN: REFERENCES

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APPENDIX

A1. Absolute Methanol/3% H_2O_2 (v/v)

Mix 25 ml 30% H_2O_2 and 225 ml of Absolute Methanol just before use.

A2. 0.1M Sodium Citrate, pH 6.0 (w/v)

Dissolve 29.4 g tri-sodium citrate (Na-citrate) in 800 ml distilled water (dH_2O), adjust to pH 6 with HCl and make up to 1000 ml.

A3. 0.01M PBS, pH 7.4

Dissolve 1.19 g of sodium dihydrogen orthophosphate (NaH_2PO_4), 1.77 g of di-sodium hydrogen orthophosphate anhydrous (Na_2HPO_4) and 0.58 g of sodium chloride (NaCl) in 800 ml of dH_2O and make up to 1000 ml.

A4. Milk Block (10% v/v)

Dilute 25 ml of fat free milk in 225 ml of 0.01 M PBS.

A5. Mayer's Haemaytoxylin

Haematoxylin	1 g
Distilled Water	1000 ml
Potassium Alum	50 g
Citric Acid	1 g
Chloral Hydrate	50 g
Sodium Iodate	0.2 g

Method:

Haematoxylin, potassium alum and sodium iodate are dissolved in distilled water by warming and stirring or allowing to stand at room temperature overnight. Chloral hydrate

and citric acid are added and the mixture boiled for 5 minutes, cooled and filtered. Stain is ready for immediate use.

A6. 90% EtOH

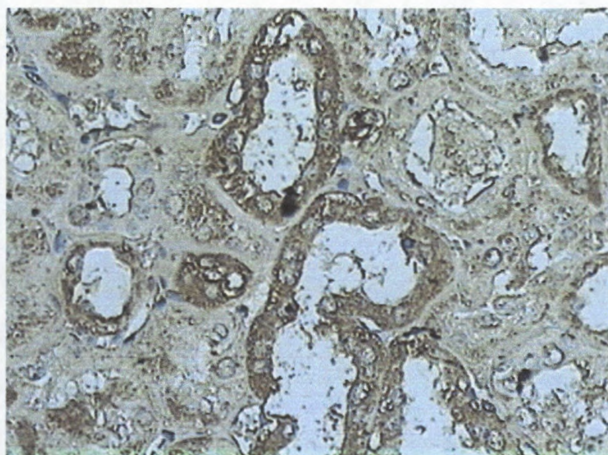
Dilute 180 ml of ethanol with 20 ml of distilled water.

A7. 70% EtOH

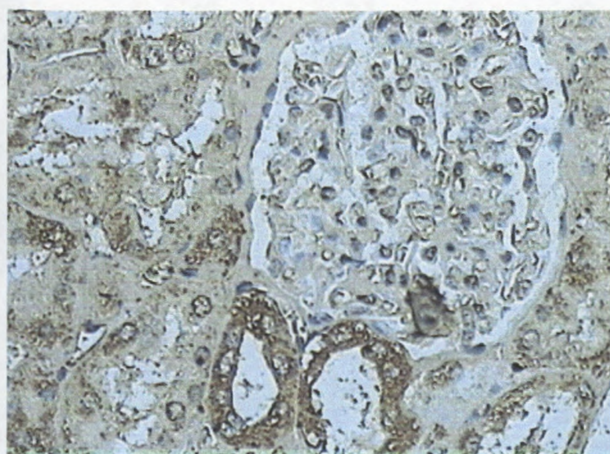
Dilute 140 ml of ethanol with 60 ml of distilled water.

A8. Additional Results

During the course of this study, 81 photographs were taken of cancerous and non-cancerous kidney sections. The numbers in parenthesis refer to samples obtained from either biopsies or post-mortem renal tissue.



Kidney Carcinoma (17173E) labelled with TK



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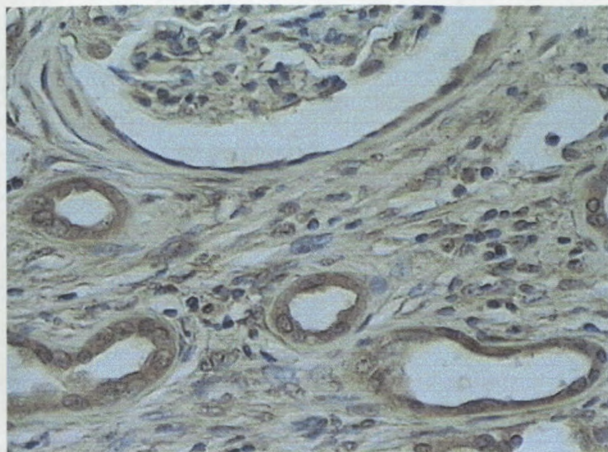
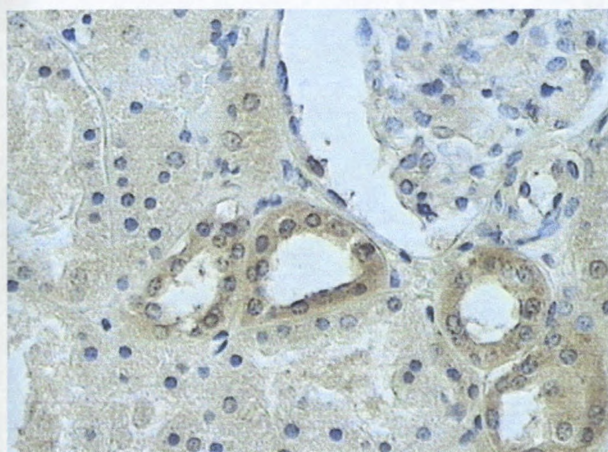
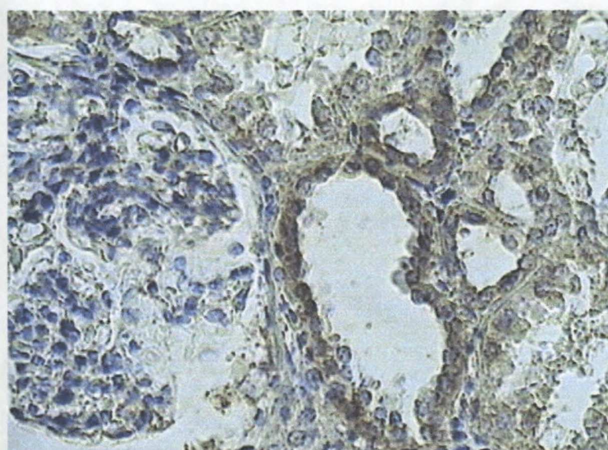


Kidney Carcinoma (RCA) labelled with TK



Kidney Carcinoma (RKIDCA) labelled with TK



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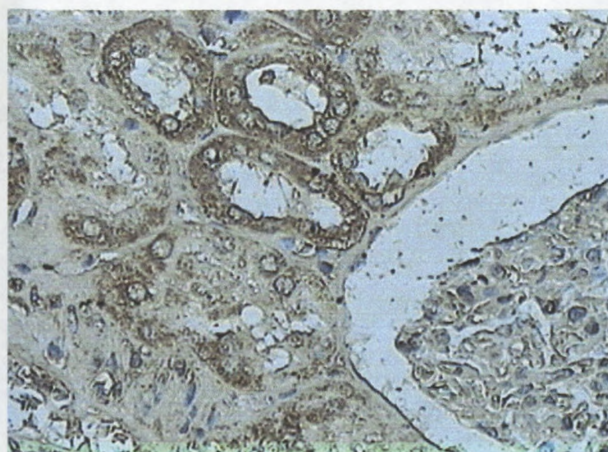

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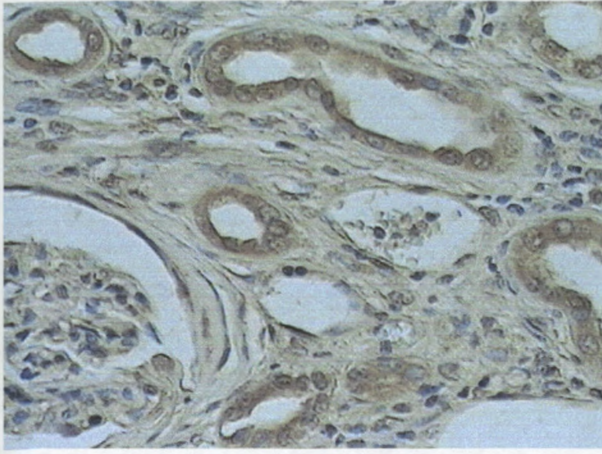

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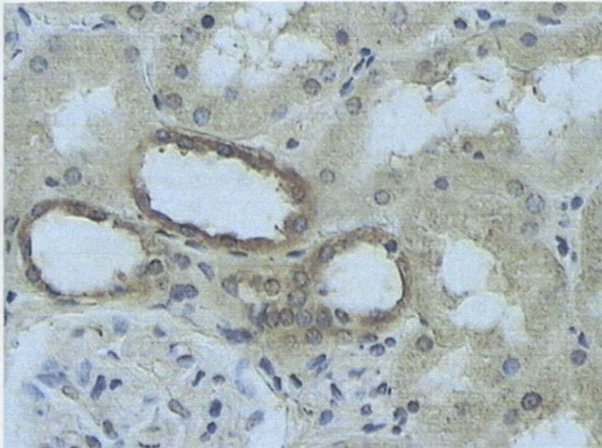

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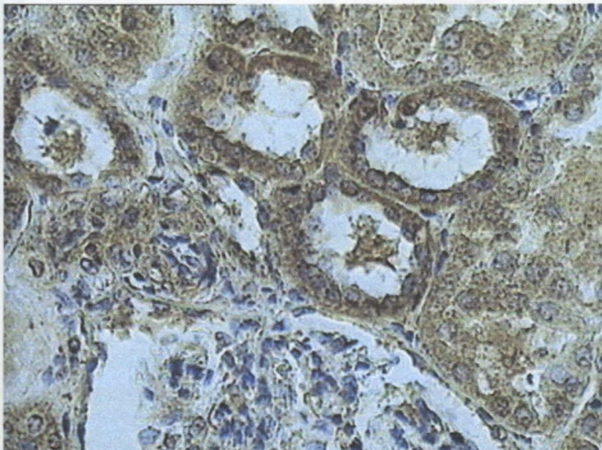
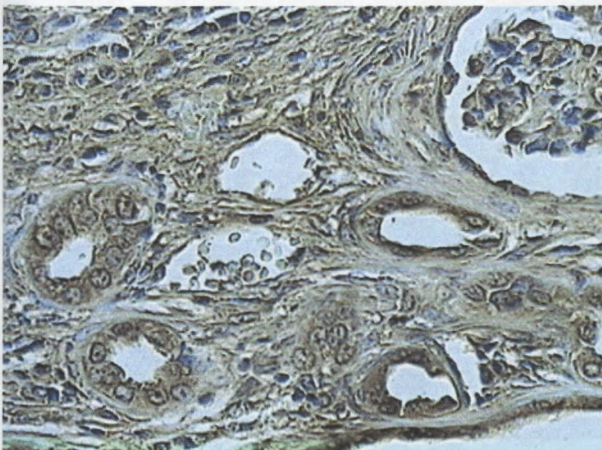
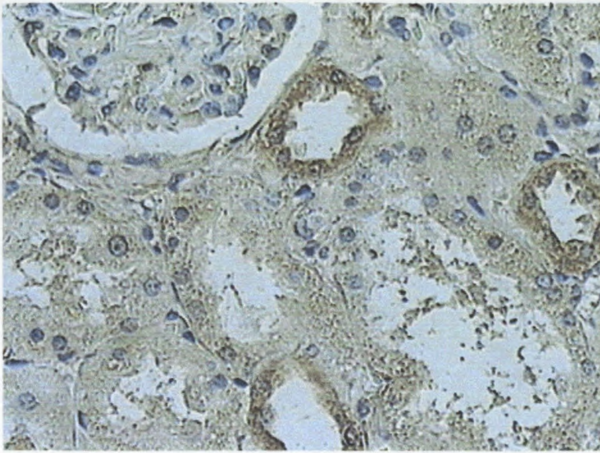
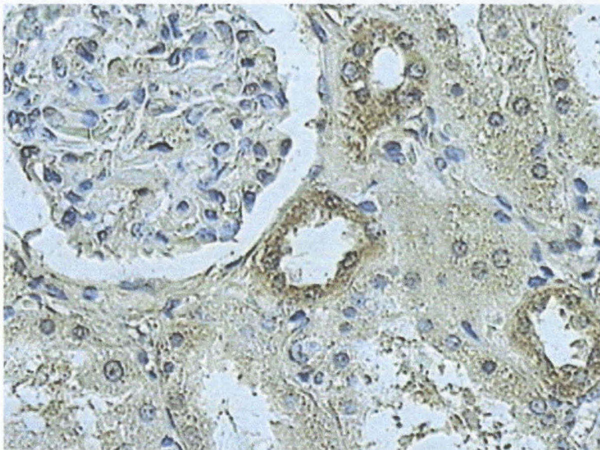


Kidney carcinoma (1003F) labelled with kinin receptor B1



Kidney carcinoma (RCA) labelled with kinin receptor B1


Kidney carcinoma (RKIDCA) labelled with kinin receptor B1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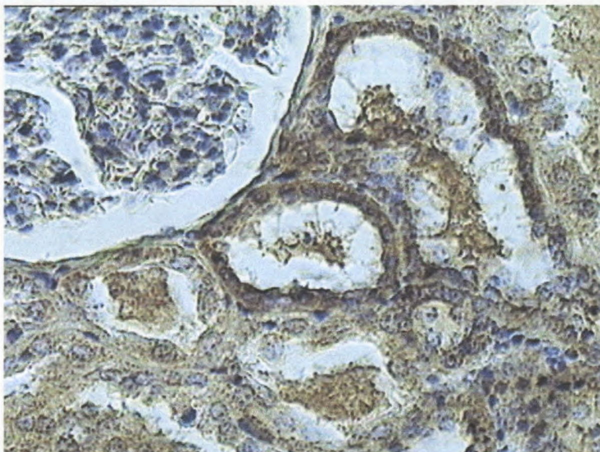
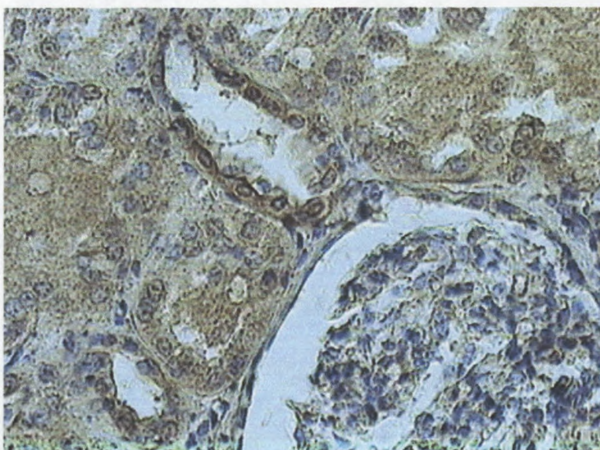
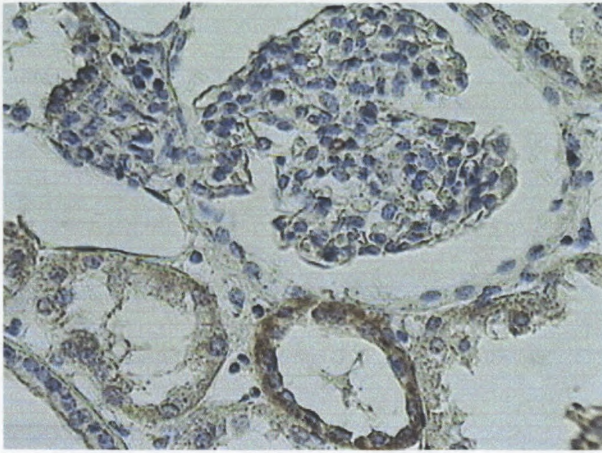
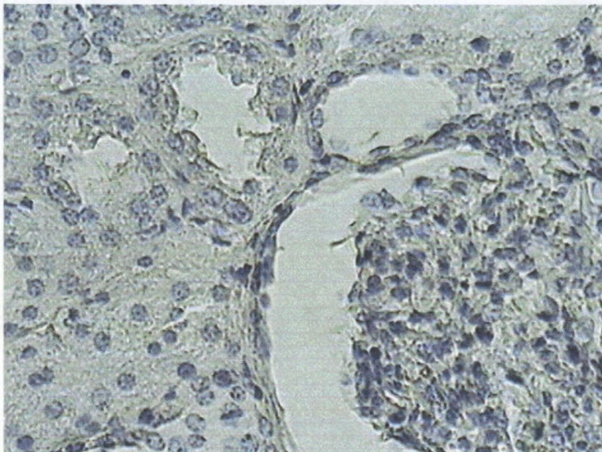

Kidney Carcinoma (1003F) labeled with kinin receptor B2

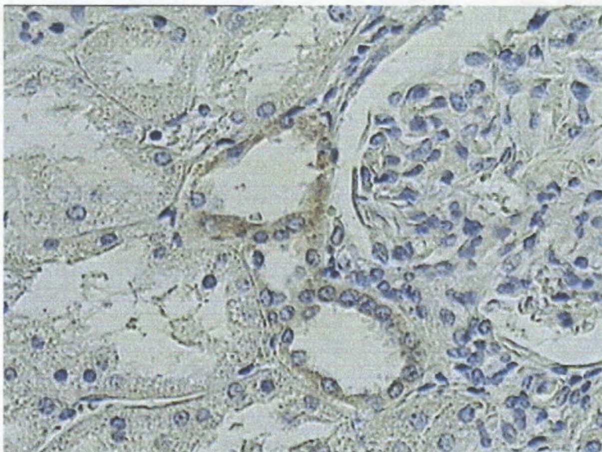

Kidney Carcinoma (1003F) labeled with kinin receptor B2



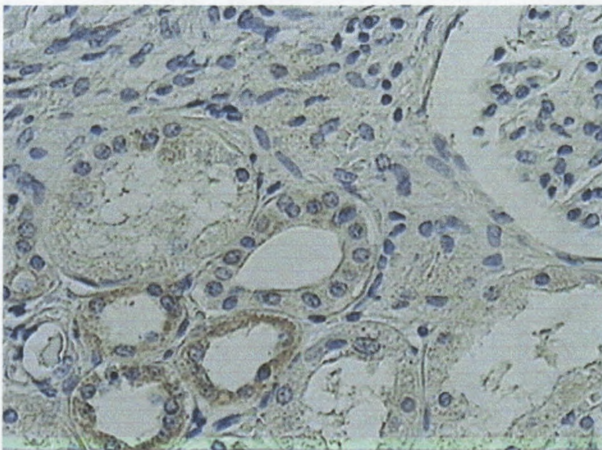
Kidney carcinoma (1003F) labelled with TGFβ Receptor II



Kidney carcinoma (1003F) labelled with TGFβ Receptor II



Kidney carcinoma (RKIDCA) labelled with TGFβ Receptor II



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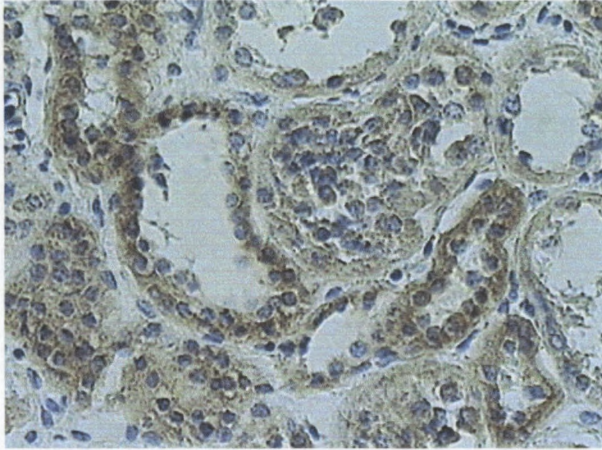
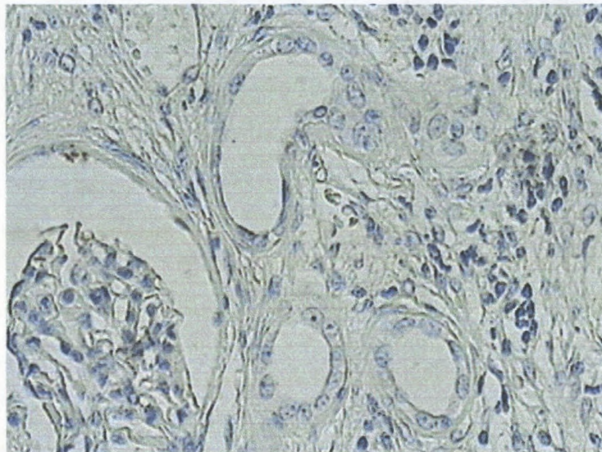
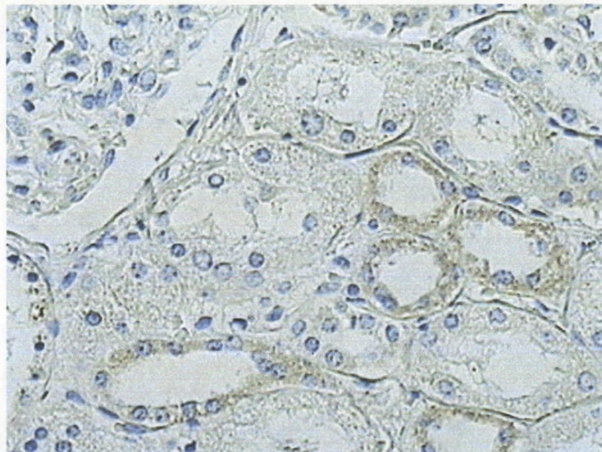


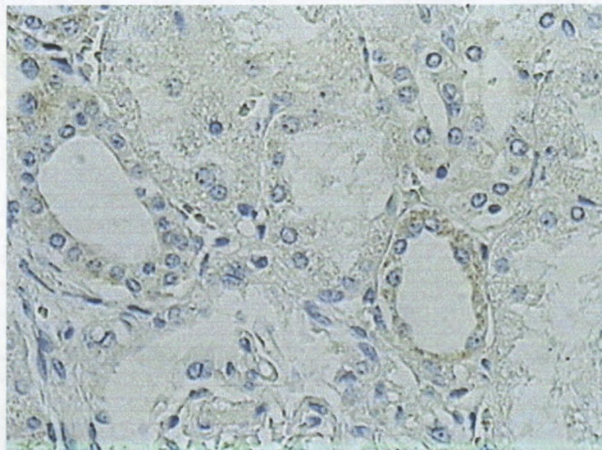
Kidney carcinoma (1003F) labeled with TGFβ Receptor III



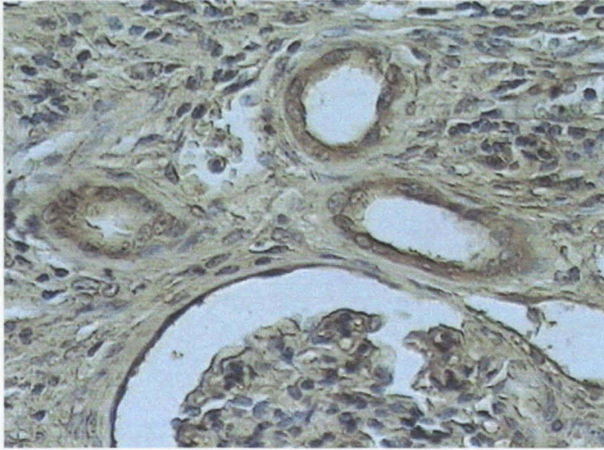
Kidney carcinoma (RCA) labeled with TGFβ Receptor III



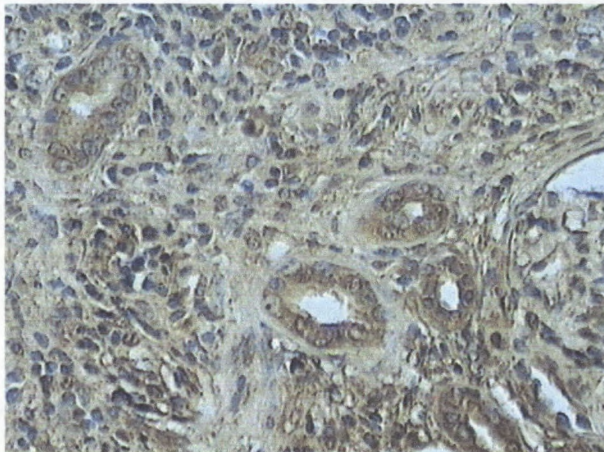
Kidney carcinoma (RKIDCA) labeled with TGFβ Receptor III



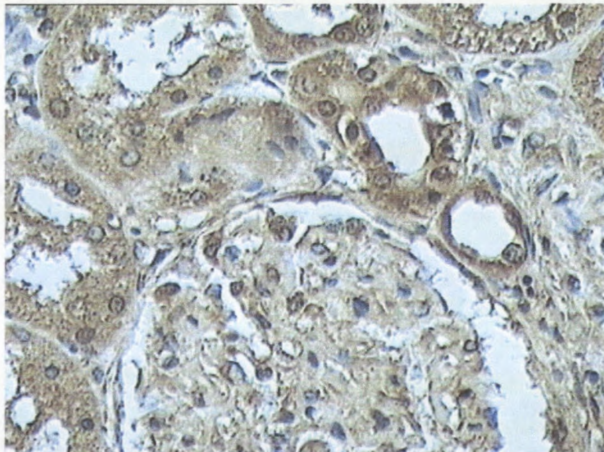
Kidney carcinoma (RKIDCA) labeled with TGFβ Receptor III



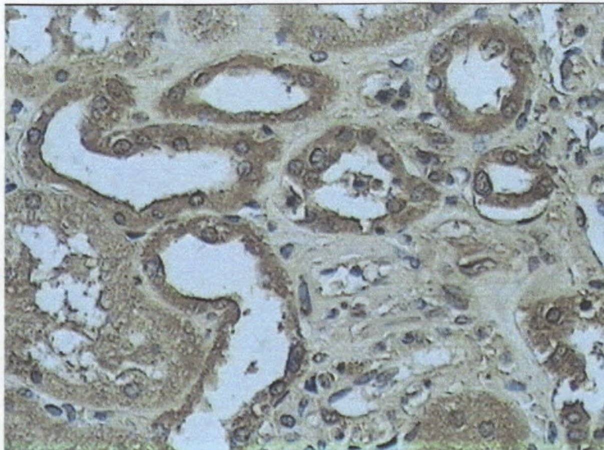
Kidney carcinoma (RCA) labeled with $TGF\beta_1$



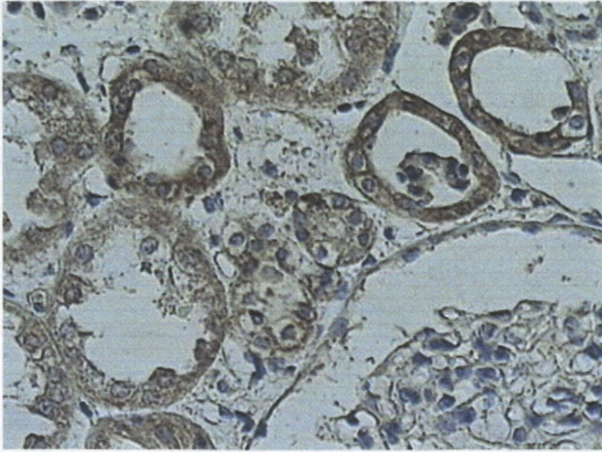
Kidney carcinoma (RCA) labeled with $TGF\beta_1$



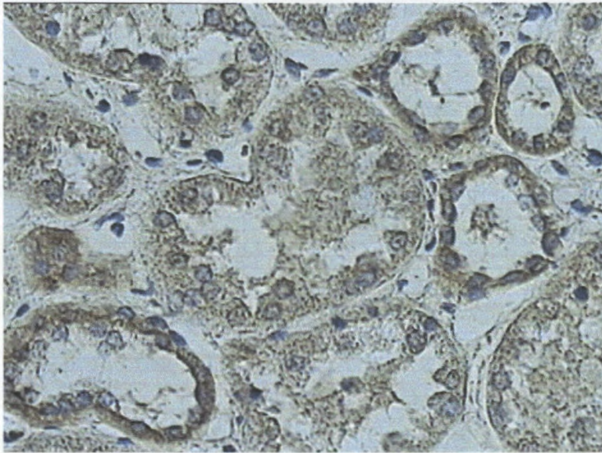
Kidney carcinoma (RKIDCA) labeled with $TGF\beta_1$



Kidney carcinoma (RKIDCA) labeled with $TGF\beta_1$



Kidney carcinoma (1003F) labeled with $TGF\beta_1$



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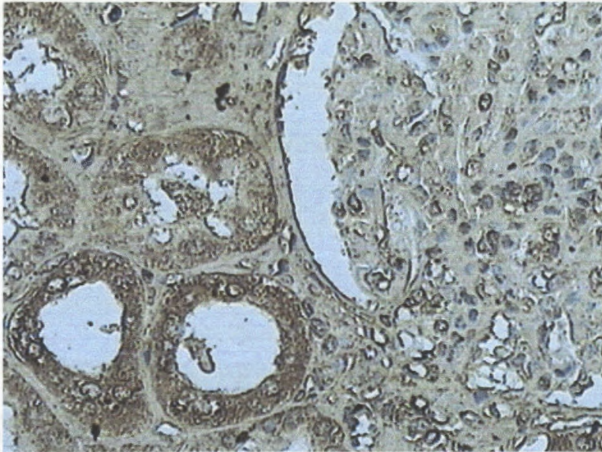
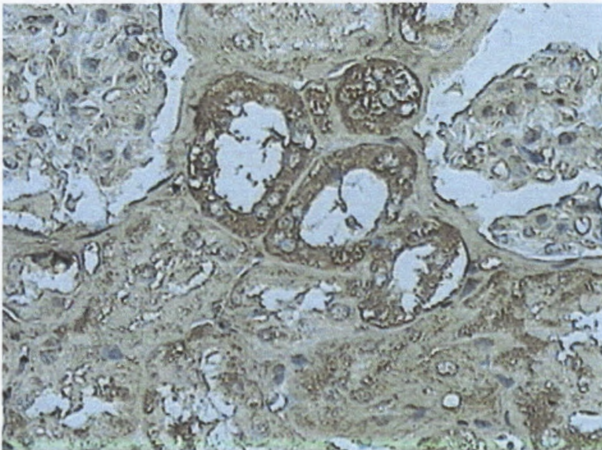


Kidney carcinoma (17173E) labeled with $TGF\beta_1$



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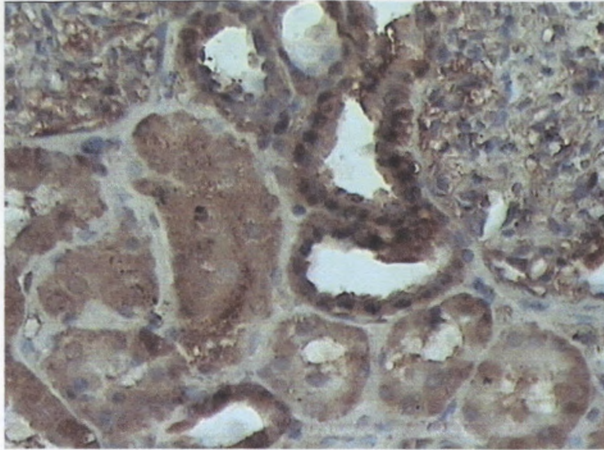
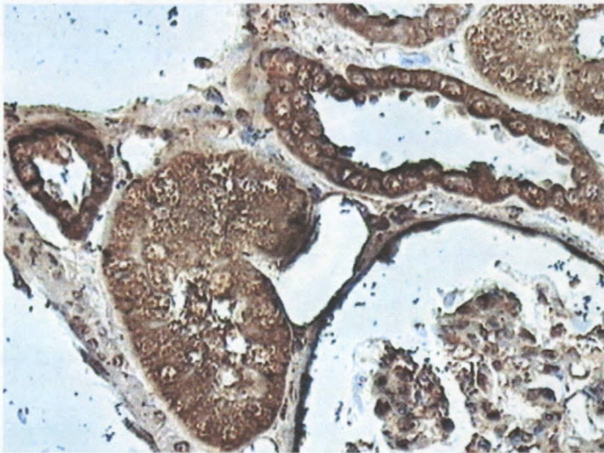
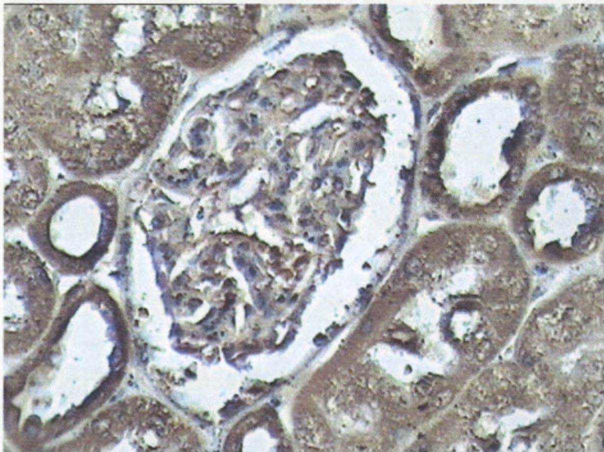


Non-cancerous kidney (NGRANT) labelled with TGFβ and TK



Non-cancerous kidney (NKID) labelled with TGFβ and TK



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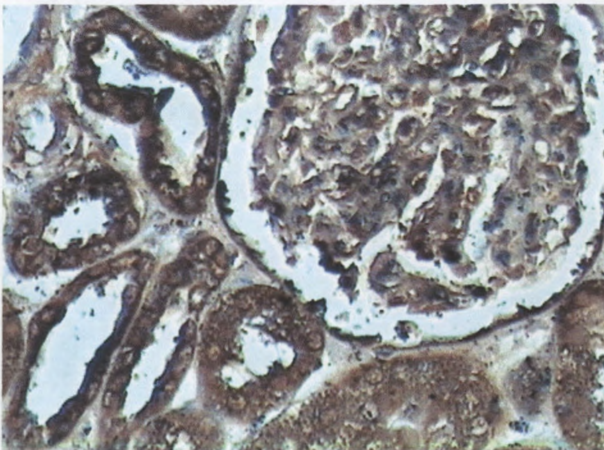
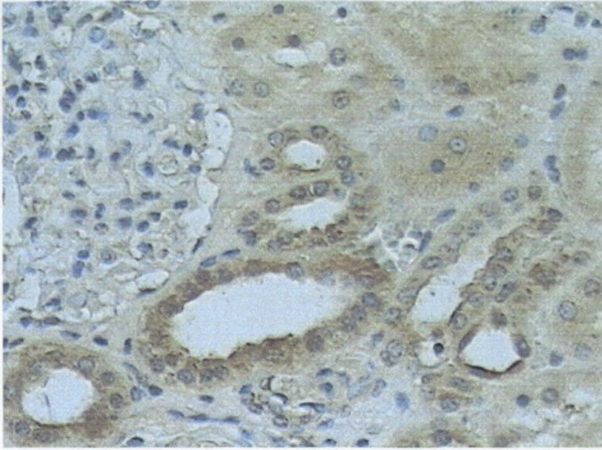
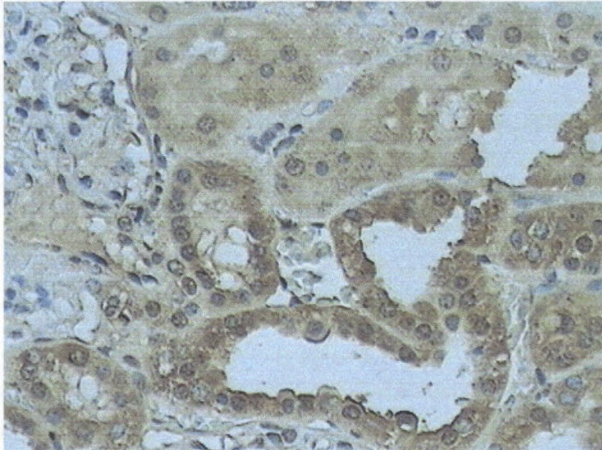


Non-cancerous kidney (NKID) labelled with TGFβ and TK



Non-cancerous kidney (NGRANT) labeled with TK



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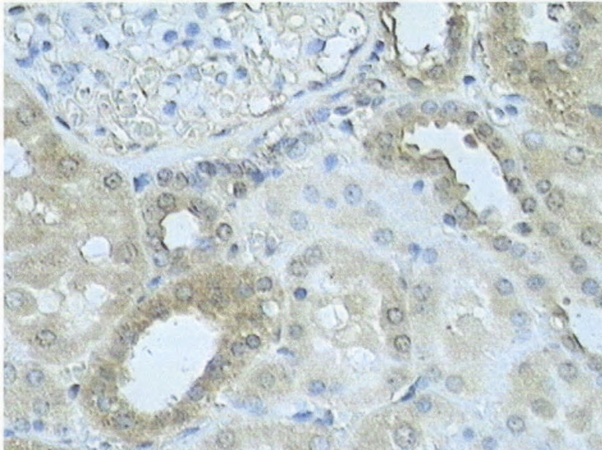

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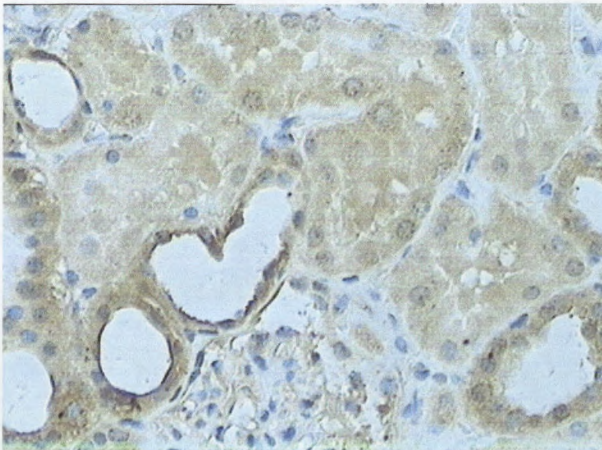
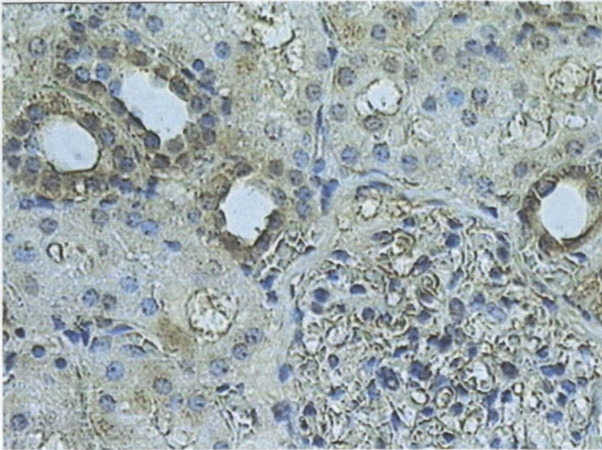
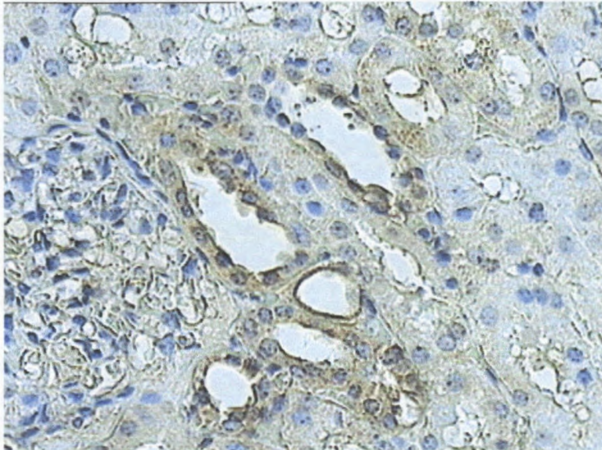
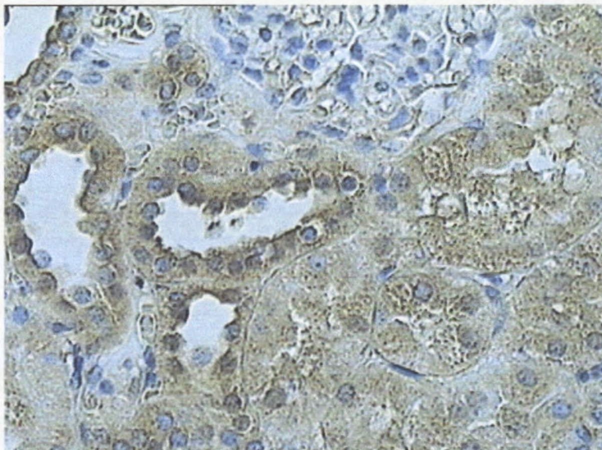
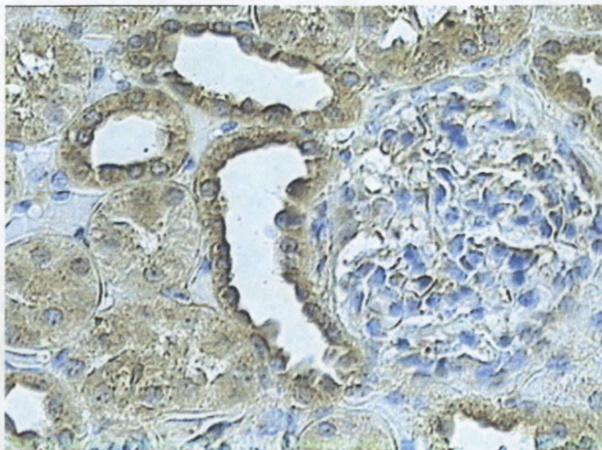


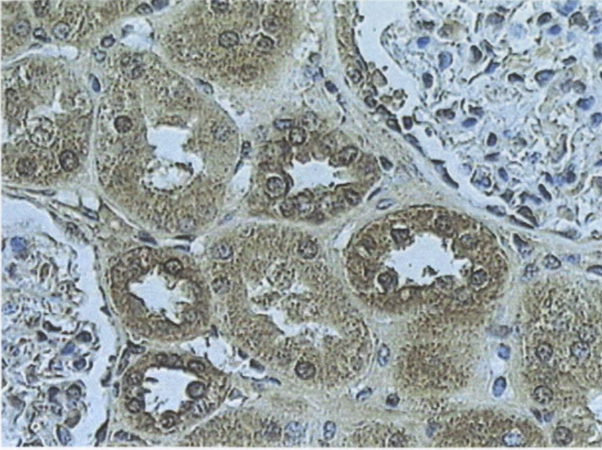
Non-cancerous kidney (NGRANT) labeled with TK


Non-cancerous kidney (NGRANT) labelled with kinin B1 Receptor

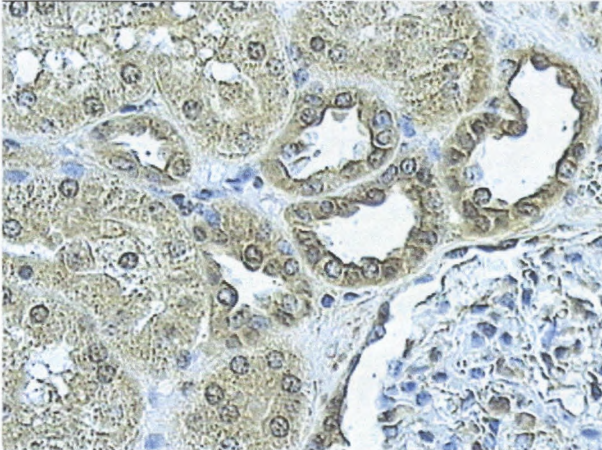

Non-cancerous kidney (NGRANT) labelled with kinin B1 Receptor


Non-cancerous kidney (NKID) labelled with kinin B1 Receptor

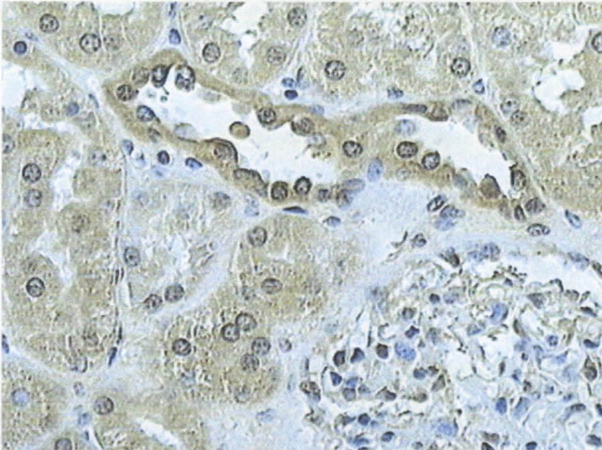

Non-cancerous kidney (NKID) labelled with kinin B1 Receptor



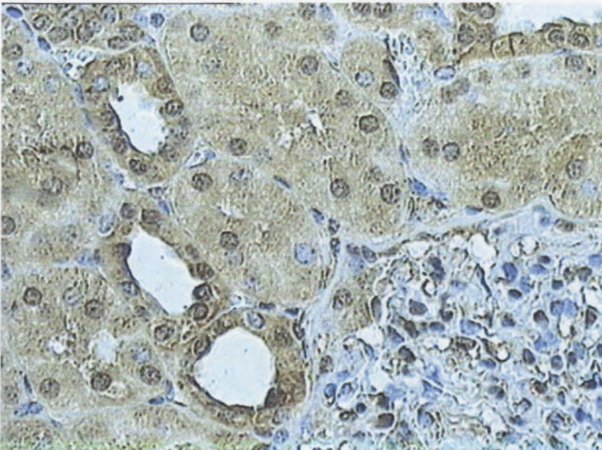
Non-cancerous kidney (NKID) labelled with kinin B2 Receptor



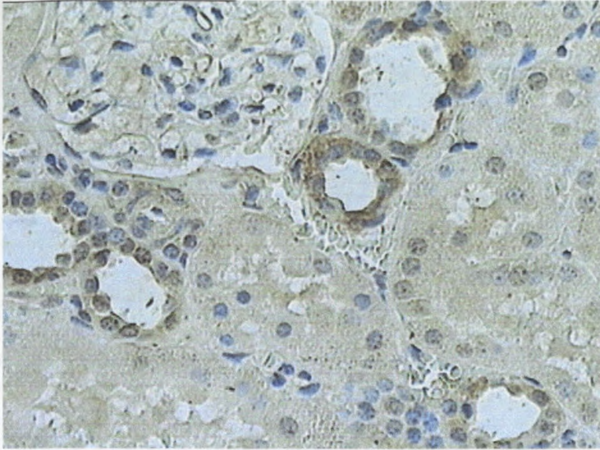
Non-cancerous kidney (NKID) labelled with kinin B1 Receptor



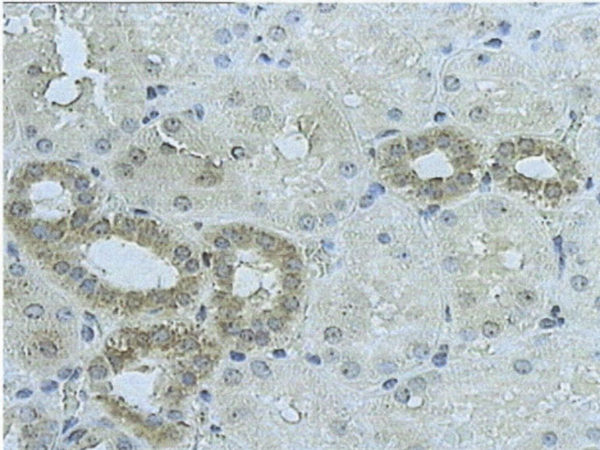
Non-cancerous kidney (NKID) labelled with kinin B1 Receptor



Non-cancerous kidney (NKID) labelled with kinin B1 Receptor



Non-cancerous kidney (NGRANT) labelled with kinin B1 Receptor



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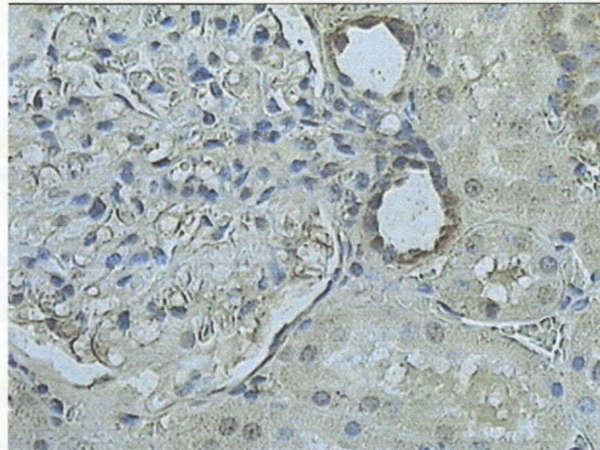
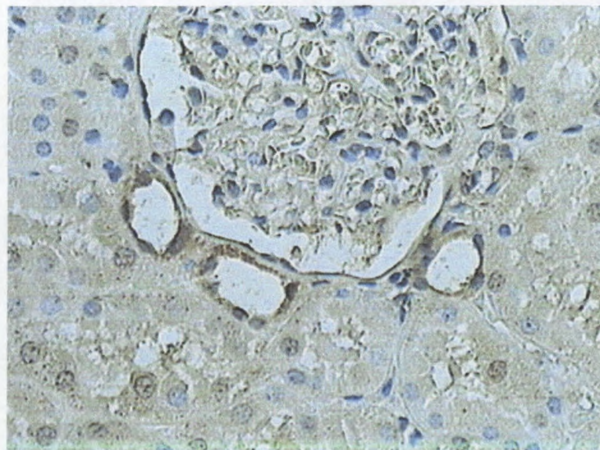
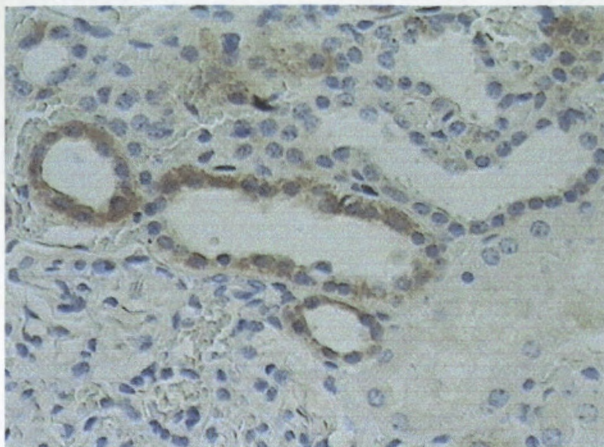


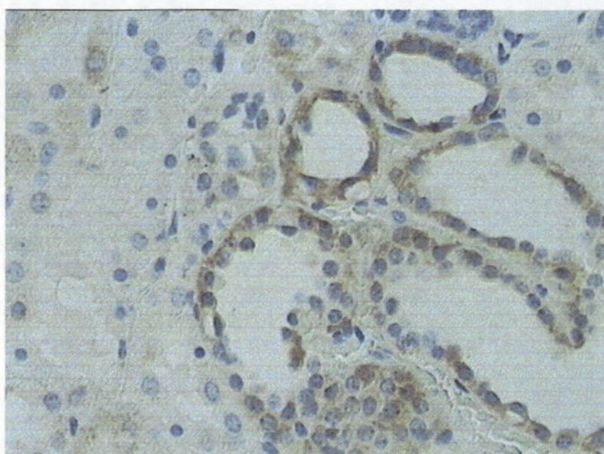
Non-cancerous kidney (NGRANT) labelled with kinin B1 Receptor



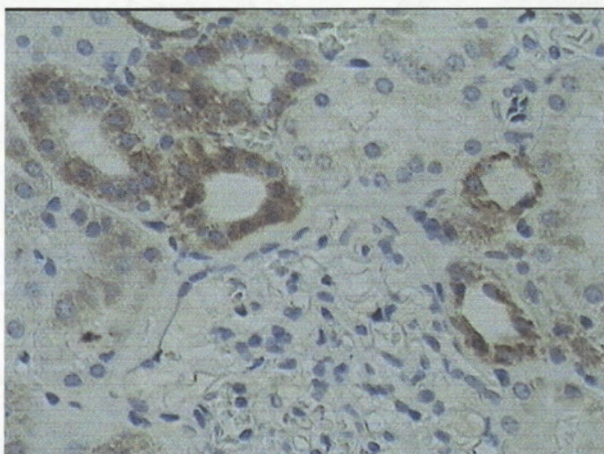
Non-cancerous kidney (NGRANT) labelled with kinin B1 Receptor



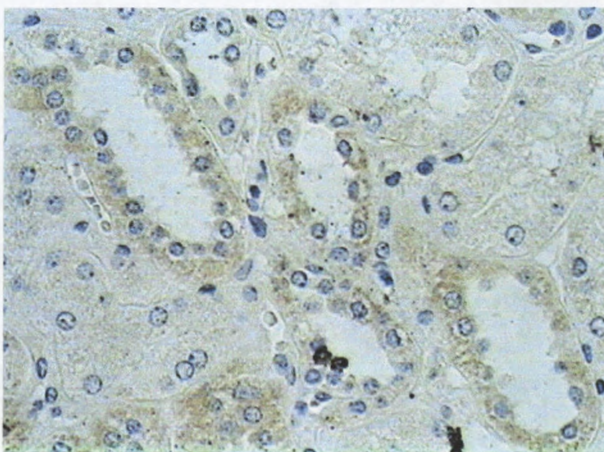
Non-cancerous kidney (NGRANT) labelled with TGFβ Receptor II



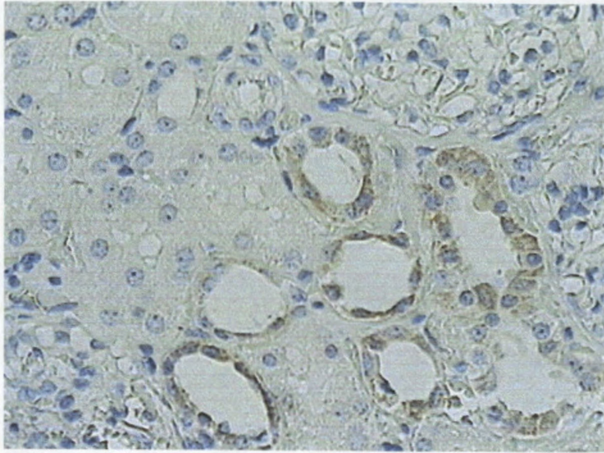
Non-cancerous kidney (NGRANT) labelled with TGFβ Receptor II



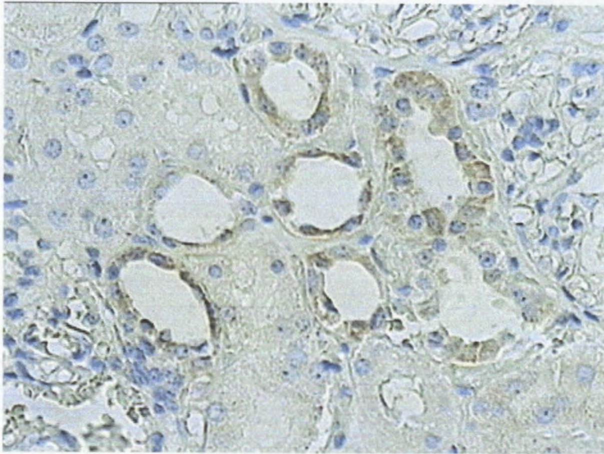
Non-cancerous kidney (NGRANT) labelled with TGFβ Receptor II



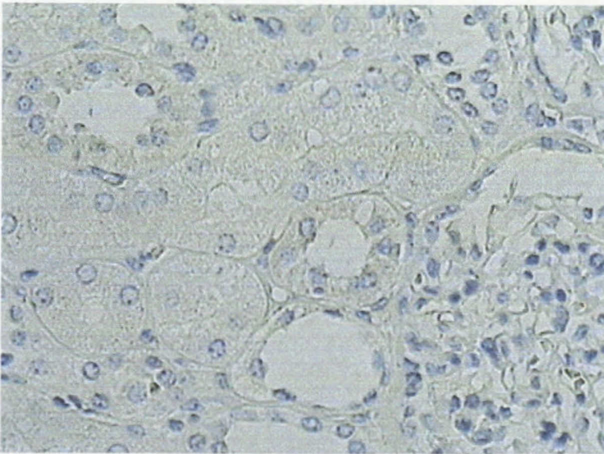
Non-cancerous kidney (NKID) labelled with TGFβ Receptor II



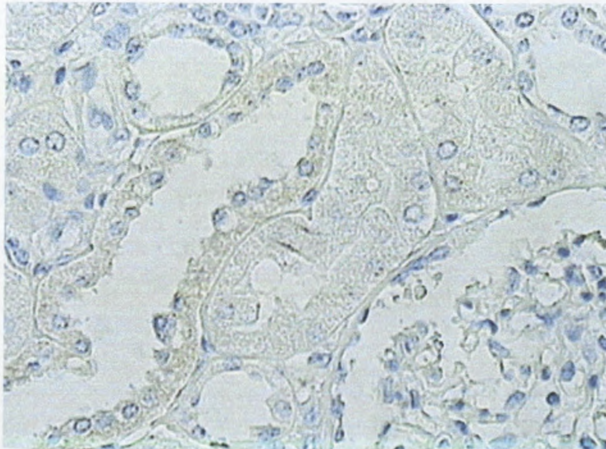
Non-cancerous kidney (NGRANT) labelled with TGFβ Receptor III



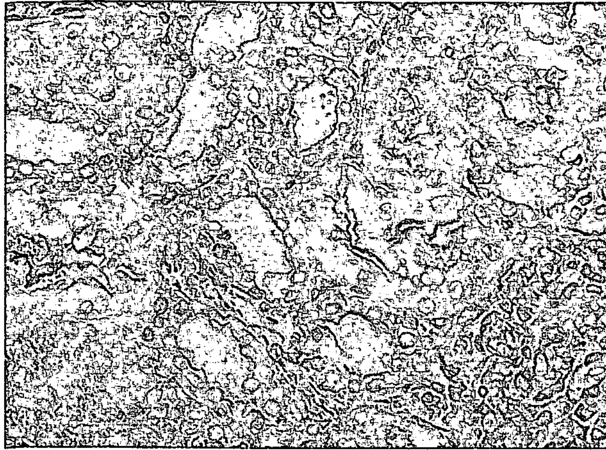
Non-cancerous kidney (NGRANT) labelled with TGFβ Receptor III



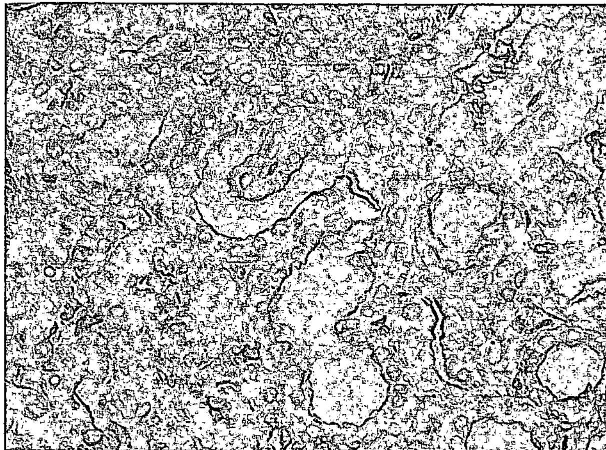
Non-cancerous kidney (NKID) labelled with TGFβ Receptor III



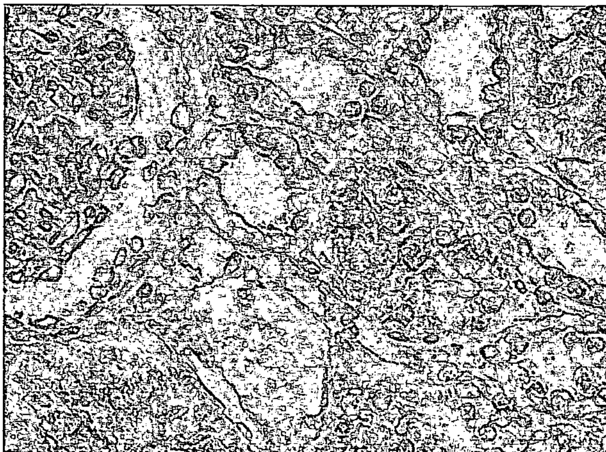
Non-cancerous kidney(NKID) labelled with TGFβ Receptor III



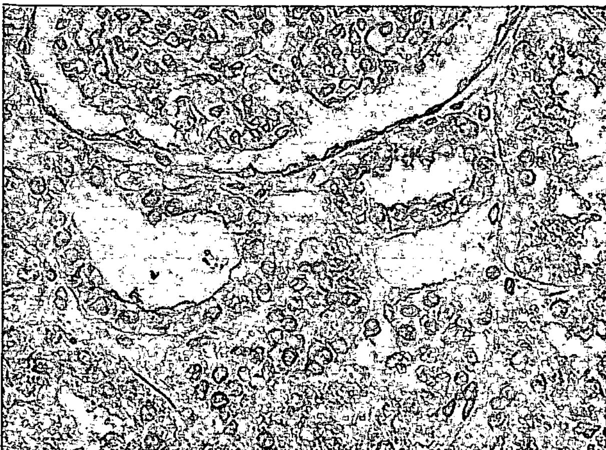
Non-cancerous kidney (NGRANT) labelled with TGFβ_1



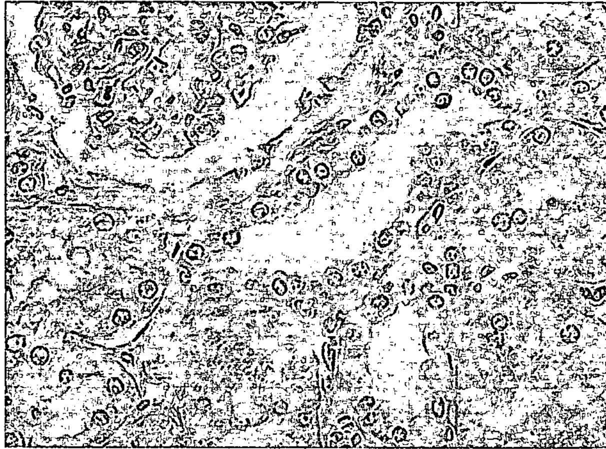
Non-cancerous kidney (NGRANT) labelled with TGFβ_1



Non-cancerous kidney (NKID) labelled with TGFβ_1



Non-cancerous kidney (NKID) labelled with TGFβ_1



Method controls (NKID)

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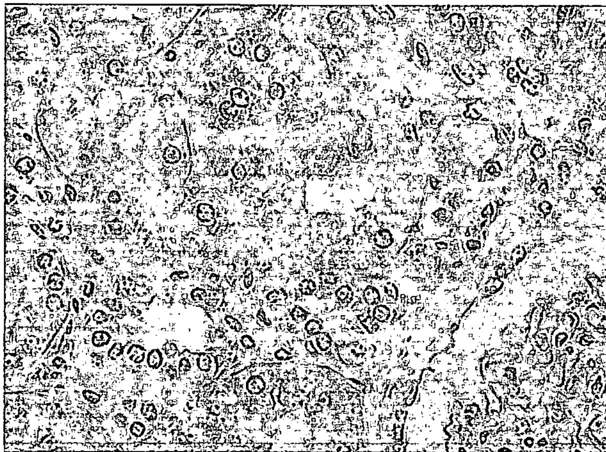

Method controls (NKID)