

Cell cycle regulatory factors in juxtatumoral renal parenchyma

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Abstract

The aim of this study was to evaluate regulatory cell cycle factors in juxta-tumoral renal parenchyma in order to obtain information regarding early primary changes occurred in normal renal cells. Specimens of juxta-tumoral renal parenchyma were harvested from the tumoral kidney in 10 patients with no history of treatment before surgery. The expression of p53, Bcl-2, Rb and PCNA was studied by immunohistochemical methods in paraffin-embedded tissues. The apoptotic status was evaluated by flow-cytometry analysis following propidium iodide incorporation. The p53 protein expression was recognized in most of the cases (80%) with different intensities. High intensity apoptotic process detected in juxta-tumoral parenchyma seemed to be p53 dependent and well correlated with the low Bcl-2 expression. 70% of cases were Rb positive. In this type of tissue Rb has only an anti-proliferative and anti-tumoral role. PCNA was present in half of the cases being low expressed due to the tissue regenerating mechanism. Our data suggest that the high intensity of programmed cell death in this type of tissue is supported by the status of cell regulatory factors that control this process. Previous studies have demonstrated that healthy renal tissue has neither apoptosis nor mitotic activity. Juxta-tumoral renal tissue is also displaying normal morphology and DNA content (diploidy) but the microenvironmental status induced by the tumor presence prompts cells to choose death rather than malignant transformation. Further studies are necessary to emphasize if these results have a clinical relevance for the outcome of therapeutical approaches in renal carcinomas.

Keywords: apoptosis, juxta-tumoral renal parenchyma, p53, Bcl-2, Rb, PCNA.

Introduction

Multiple genetic changes occur during the evolution of normal to cancer cells. This evolution is facilitated in cancer cells by the loss of fidelity in the processes that replicate, repair and segregate the genome. Many features of neoplastic cells like the loss of differentiation capacity, invasion increase and sensitiveness decrease at drugs are different from those of normal cells, and probably result from evolution process of the cell [1].

Apoptosis was described in association with embryogenesis and also in many renal diseases. It has a positive role in renal unit morphogenesis; in adult kidney is important to maintain the tissue homeostasis, in repair process as reply at decrease oxygen supply or nutrient or therapeutically induced in neoplasms [2].

Apoptosis play a central role in genitor-urinary tract development. Bcl 2 seems to be essential for interaction between epithelial uretheric burgeons and methanephric blastem that results in renal tube and collecting system formation as long as caspases are functional [3].

In adult benign urological diseases, low renal chronic ischemia leads to diffuse renal cell apoptosis with the loss of kidney parenchyma mass and function. Obstructive nephropatia suppress EGF (epithelial growth factor) expression in epithelial cells from renal tubes followed by apoptosis [4]. Moreover, apoptosis increased in human polycystic kidney disease [5].

In malignant urological disease the resistance to

apoptosis is an obvious feature of carcinogenesis and underlines the tumor resistance to classical treatments.

Many characterized oncogenes and tumor suppressor genes encode for proteins with apoptotic function (especially p53 and bcl-2).

At present, therapeutical induction of programmed cell death is achieved by using unspecific cytotoxic agents like cytostatic drugs and radiotherapy.

Pro-apoptotic peptides were recently synthesized in order to target specifically the malignant cells, this being the prologue of safe clinical induction of apoptosis [6].

Renal cell carcinoma is one of the most unpredictable human malignant diseases. Survival after 5 years from total nephrectomy ranges between 30-60% and metastatic potential can occur even after 13 years [7]. Renal cell carcinoma is characterized by a long local growth period without any clinical symptoms. It is generally diagnosed in a late evolution state when haematuria and other changes appear [8].

Although many progresses were made in the understanding of renal carcinoma biology, due to the in vitro and in vivo studies, there remain some questions without answer concerning precursor malignant lesions.

Unforeseen clinical behavior of renal carcinoma requests other pathological parameters to be investigated, in addition to tumor stage and grade which are already established to be prognostic parameters of this neoplasm [9].

Pediatric and adult studies of renal cell carcinoma allowed to identify several alterations within the genes involved in apoptosis control mechanisms, suggesting that disturbance of this process could have a role in the development of kidney cancer.

Although it is generally accepted that kidney is a "silent" organ, with no apoptotic or mitotic activity [10], there are studies which demonstrate that normal kidney cells could undergo apoptosis in some physiological or pathological conditions. In some circumstances renal cell apoptotic answer may be destructive and lead to drastic loss of kidney function (e.g. ischemic and toxic injury), but the main benefit of apoptotic process activation versus necrosis mechanism could be represented by the decrease of inflammatory answer in the tissue [2].

Apoptotic tendency of normal kidney cells is induced by the paucity of growth factors, stress factors exposition, lipid peroxide inhibition or oxygen radical generation [11].

It is also accepted the fact that apoptosis plays an important role in cell loss and in acute and chronic renal failure. In the polycystic kidney renal cells undergo apoptosis in ischemia, obstruction and compression absence, suggesting that apoptosis may be an inherited feature of renal cells [12].

In normal tissue, programmed cell death in conjunction with mitosis play a main role in maintaining tissue homeostasy [13], and neoplastic pathology is closely related with the unbalance between apoptosis and cell proliferation.

The study of juxta-tumoral renal parenchyma could give information regarding early primary changes which occurred in normal renal cells and might lead to malignant change. After our knowledge, few data concerning the evaluation of the involvement degree and pattern of cell cycle regulatory factors in renal juxta-tumoral cell apoptosis are available.

Thus, there are analyzed several proteins with major implications in maintaining the balance between cell proliferation (PCNA – proliferating cell nuclear antigen, Rb – retinoblastoma) and apoptosis (p53 and Bcl-2) whose disturb leads to tumor occurrence and progression.

☐ Material and methods

Patients: Resected human kidney specimens were provided by the Department of Urology, "Prof. Dr. Th. Burghel" Clinical Hospital Bucharest, Romania. The study was carried out on 10 cases of renal carcinomas with no history of chemotherapy, radiotherapy or immunotherapy. Half (50%) of the cases were males and half were females with an age range between 41 and 76 years.

Cells: Specimens of juxta-tumoral renal parenchyma were harvested from tumoral kidneys, as far as possible from the tumor site.

Freshly isolated tissue explants were obtained within 15 min of devascularization and maintained on ice in sterile complete culture medium (CCM), represented by RPMI-1640 medium supplemented with 10% fetal calf

serum (FCS), 2mM L-glutamine, and antibiotics (penicillin and streptomycin) (Sigma, St. Louis, MO).

Single cell suspensions were obtained by tissue mechanic processing and enzymatic digestion using 0.4mg/ml collagenase V, 0.1 mg/ml hyaluronidase I-S and 0.2 mg/ml deoxyribonuclease (Sigma) in RPMI-1640 medium.

The cell suspensions were then filtered through 50 mesh nylon sieve, to remove cell clumps and undigested tissue fragments and washed in CCM. Single cell suspension was verified by Giemsa staining and light microscopy. Cell viability was tested by trypan blue exclusion.

Evaluation of apoptosis by PI incorporation: Apoptosis analysis was performed by staining the single cell suspension with 50 µg/ml of propidium iodide (PI) (Boehringer Corp. Mannheim, Germany) in 0.1% sodium citrate and 0.1% Triton X-100 according to Nicoletti's protocol [14]. Data analysis was performed using WinMDI 2.8 PC software.

Immunohistochemistry: Immunohistochemical staining was performed using the avidin-biotin peroxidase complex method, as described previously [15]. Briefly, 5µm sections were placed on poly-L-lysine-coated slides. All tissues were deparaffinized using standard techniques. The sections were boiled 10 minutes in 10mM citrate buffer (pH 6.0) for antigen retrieval. After a blocking step with 1.5% hydrogen peroxide in methanol for 10 minutes the sections were incubated in avidin and biotin for 20 minutes each, followed by 3% normal sheep serum at room temperature.

The sections were then incubated overnight at 4°C in a humidified chamber with mouse anti-human monoclonal antibodies: anti-p53 (clone DO-7 Dako A/S, Denmark), anti-Bcl-2 (clone 124, Dako), anti-Rb (clone G3-245, Pharmingen, Germany) and anti-PCNA (clone PC-10, Dako). Titer runs were performed to optimize staining. The sections were then incubated with biotinylated goat anti-mouse IgG (Sigma) for 30 minutes. After incubation with ABC complex-HRP (Dako) for 45 minutes samples were exposed to diaminobenzidine tetrahydrochloride (DAB) solution and contra-stained with Mayer's hematoxylin. After dehydration in progressive concentrations of ethanol and xylene, sections were mounted in Canada balsam (Sigma).

Scoring: Two observers who were blinded to the clinicopathological characteristics of the patients assessed the evaluation of staining. All tissues were scored for overall tissue expression as follows: 0, no staining; 1+, 1% to 25% staining; 2+, 26% to 50% staining; 3+, 51% to 75% staining and 4+, 76% to 100% staining.

☐ Results and discussion

The percentages of apoptotic events detected in renal juxta-tumoral tissue displaying normal morphology and DNA content (diploidy) were high in almost all the investigated cases (mean% ± SD = 80.5 ± 22.14). Data support the results published in a previous study [16].

Figure 1 – p53, intense positive reaction at epithelial tubular cells level (ob. ×20) (case no. 2)

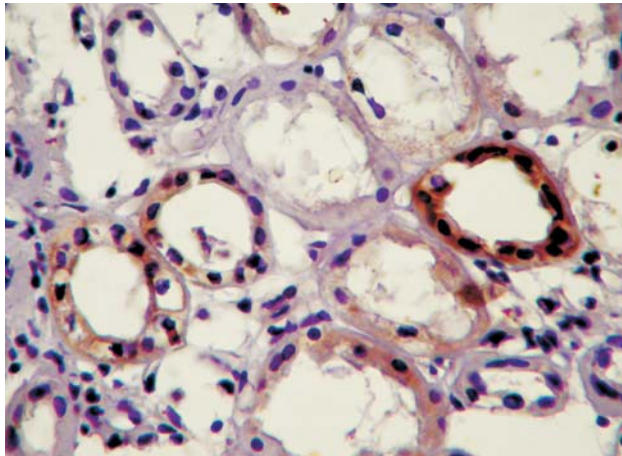
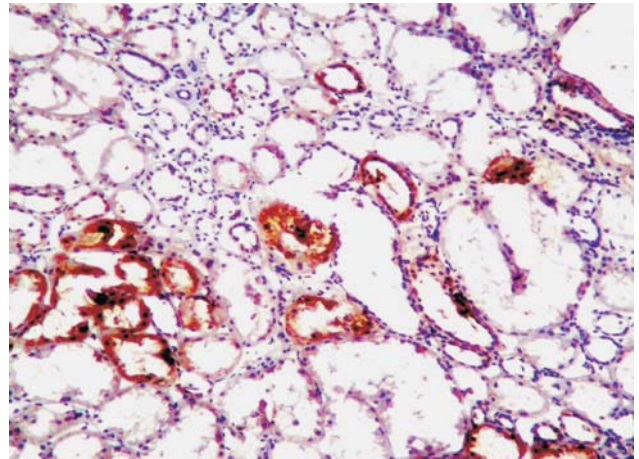


Figure 2 – Bcl-2, positive reaction in epithelial distal tubes cells (ob. ×40) (case no.4)

Figure 3 – Rb, positive reaction in peritubular cells (ob. ×10) (case no.3)

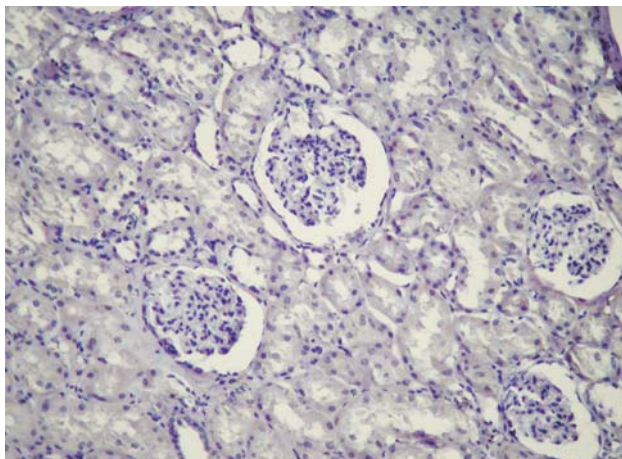
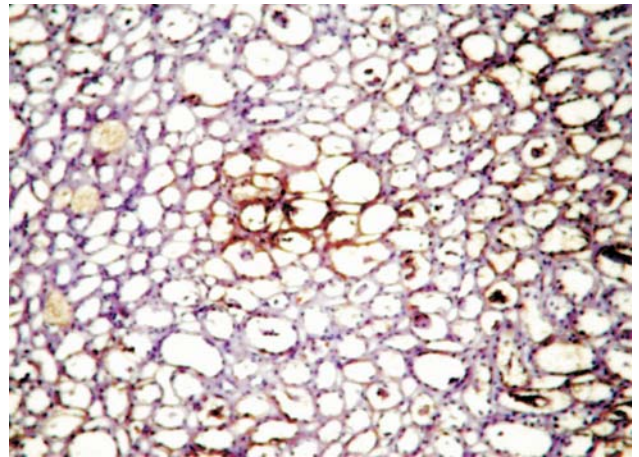


Figure 4 – PCNA, negative reaction in all cells (ob. ×20) (case no. 5)

An explanation for the high spontaneous apoptotic level, detected in the juxta-tumoral tissue could be the presence of ischemia, knowing that the kidney is an extremely sensitive organ at oxygen absence.

Moreover, other microenvironmental factors, like growth factors depletion, cell starvation due to the growing necessities of the tumor lying at the opposite pole of the organ, could determine juxta-tumoral parenchyma undergo programmed cell death.

Apoptosis induction could be also due to the instability of genomic expression of the apparently normal cells, state that could determine the cell to commit "suicide", auto-destruction rather than malignant transformation. A normal cell can respond to DNA-damage in few different ways: it can stop cell division until the damage is repaired, undergo apoptosis or enter the cell cycle without any interruption leading to malignant transformation [17].

Apoptotic DNA fragmentation minimizes the risk of transferring genetic information from apoptotic cancer

cells to the neighboring cells, either tumor or healthy [18].

P53 is a tumor suppressor protein, involved in the cell cycle control, which allows cellular DNA repair and/or apoptosis to occur by controlling cellular progression from the G1 to the S phase [19].

Immunohistochemical analysis shows p53 oncoprotein in 80% of the cases with different intensities, being relatively homogenous distributed between 1 and 75%. The intensity of apoptotic process as detected by flow-cytometry was extremely high in most of the cases, enrolling between 75–100% (Table 1).

P53 protein expression was mainly observed in tubular epithelial cells, intense in interstitial and rare in glomerular cells (Figure 1).

P53 protein intensity was mainly associated with very high values of apoptosis. The p53 immunohistochemical staining showed nuclear localization, but it was also present in the cytoplasm.

Table 1 - Oncoproteins expression detected by immunohistochemistry in renal juxtatumoral tissue harvested from patients with different renal cancer histotypes

Case no.	Apoptosis [%]	P53	Bcl-2	Rb	PCNA	Tumor Cell Type
1.	89	+	+	++	++	Clare cell carcinoma
2.	97	++	++	++	+	Cromophobe cell carcinoma
3.	98	+++	+	++	+	Clare cell carcinoma
4.	90	+	+	-	-	Clare cell carcinoma (clare cell + eosinophilic cell)
5.	55	+	+	-	-	Clare cell carcinoma carcinoma
6.	76	+	-	+	+	Clare cell carcinoma (clare cell + fusiform cell)
7.	90	-	-	+	-	Papillary carcinoma
8.	85	+++	+	+	++	Transitional cell carcinoma
9.	96	++	+	+	-	Transitional cell carcinoma
10.	29	-	-	-	-	Transitional cell carcinoma

Our data regarding p53 tumor suppressor protein expression in apparently normal renal tissue lead to the conclusion that in this case the apoptotic process seems to be p53-dependent.

Hipoxia represents a factor capable to stimulate p53 expression. This process may represent another way though which p53 protein acts as a protector against cancer occurrence.

A lot of tumors start to grow and reach a critical size until sanguine influx becomes limited, needing increased oxygen supply which can support the growing. The resulting hipoxia may stimulate p53 activity and kill tumor cells.

Absence of p53 expression was found in two of the cases under study. In one case (patient no. 10) the lack of p53 expression correlates with a very low percentage of apoptotic events (29%). In the second case (patient no. 7) the high intensity of apoptotic process might be due to other inductive mechanisms that does not involve p53 oncoprotein participation.

Bcl-2 is a proto-oncogene known to be a negative regulator of apoptosis which plays an important role in cell longevity preventing programmed cell death [20].

We have detected Bcl-2 oncoprotein in 70% of the studied cases with low intensity 1-25% (Table 1), excepting one case (patient no. 2) who had a medium expression of this protein.

The absence or the low intensity expression of Bcl-2 protein was well correlated with the presence of a high percentage of apoptotic juxta-tumoral renal cells. An explanation for Bcl-2 presence in some cases might be its possible role in counterbalancing the cell loss and providing homeostasia in the untransformed tissue, while p53 promotes the suicide of the cells with malignant potential.

In non-tumoral renal tissue Bcl-2 is well expressed in glomerular epithelial cells and in epithelial cells of distal renal tubes. Generally, the proximal tubular cells were Bcl-2 negative (Figure 2).

Our data concord with those reported by Huang *et al.* who observed that normal kidney was Bcl-2 positive especially within the cytoplasm of distal tube cells. They have also observed a rare and minor staining of the proximal tube cells, supposed to be the origin of renal carcinoma, both in normal controls and juxta-tumoral areas from patients [21].

The single investigated case in which Bcl-2 expression was associated with a medium intensity of apoptosis (case no. 5) might be able to be a situation in which Bcl-2 exhibits its anti-apoptotic role and supports the improvement of programmed cell death process.

Gobe *et al.* analyzing the relationship between the expression of Bcl-2 family members, representative for pro-apoptotic (Bax) or anti-apoptotic (Bcl-2 and

Bcl-XL) functions, the incidence of apoptosis and mitosis in a selected group of 22 graded renal cell carcinomas and also in non-neoplastic tissue. The results of this study were different from ours: when Bcl-2 and/or Bcl-XL expression were high, apoptosis was not detected and when expression of this protein was low or not found increased levels of apoptosis were seen [22].

The retinoblastoma (Rb) gene product is a nuclear phosphoprotein involved in cell proliferation. Rb gene mutations or absence lead to uncontrolled replication, participating to the neoplastic phenotype occurrence [23]. Different intensities of Rb tumor suppressor were detected in 70% of the patients taken under study (see Table 1). Its expression is associated to augmentation of apoptotic intensity from apparently normal renal tissue.

The last one belongs to an organ that exhibits a malignant proliferation and the presence of Rb protein was expected in untransformed area because of its anti-proliferative and anti-tumoral roles. It is supposed that in this type of tissue Rb exhibits the anti-proliferative role because we did not detect an increase of the cell cycle S phase or a high PCNA expression (data not shown).

Moreover, Rb did not exhibit its anti-apoptotic role since the intensity of apoptotic process was very high (or reached the tardy form of apoptotic corps detection). Rb protein expression in apparently renal tissue was mainly observed in peritubular (Figure 3) and intraglomerular cells.

The proliferating cell nuclear antigen is synthesized during G1 late phase and S phase of the cell cycle. Some authors sustain that a low PCNA index (< 10%) is an independent positive predictor of survival [24]. PCNA protein was detected in half of the cases with low intensity (1-25%), and in only two cases with medium intensity (Table 1).

Since kidney is a „silent” organ from the proliferative point of view, the lack on PCNA protein expression (Figure 4) was predictable. PCNA low levels evaluated by IHC and corroborated with percentages of distribution of cells in different cell cycle phases as detected by flow-cytometry studies (data not shown) support the idea that the proliferative process is within normal limits.

In apparently normal renal tissue PCNA expression was detected as a sporadic staining of glomerular cells, being absent in tubular cells and having high expression in interstitial cells.

All these data lead to the assumption that a tissue regenerative mechanism appeared to counteract the cell suicide by which the cells with malignant potential are eliminated.

☐ Conclusions

Our results suggest that the high intensity of programmed cell death in renal juxta-tumoral tissue is supported by the status of cell regulatory factors that control this process. Apoptosis might appear because of the physical pressure exerted by the adjacent tissue hyperplasia.

The apoptosis regulators seem to have a significant role in early primary changes which occur in normal renal cells. When those are abnormal expressed, they could facilitate the susceptibility of some cells to abnormal proliferation.

Previous studies have demonstrated that healthy renal tissue has no apoptosis either mitotic activity. Juxta-tumoral renal tissue is also displaying normal morphology and DNA content (diploidy), but the microenvironmental status induced by the tumor presence induces cells to choose death rather than malignant transformation.

Further studies are necessary to emphasize if these results have a clinical relevance for possible immunotherapeutic procedures in renal carcinomas, also correlated with tumor histotype.

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