Nephrol Dial Transplant (2010) 25: 373–380
doi: 10.1093/ndt/gfp525
Advance Access publication 13 October 2009

An *in-vitro* tumour microenvironment model using adhesion to type I collagen reveals Akt-dependent radiation resistance in renal cancer cells

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Abstract

Background. Renal cell carcinoma (RCC) is considered resistant to ionizing radiation. Recently, the extracellular matrix (ECM) has been shown to play a role in both drug resistance and radiation resistance (RR). While fibronectin has been extensively investigated in the context of RR, the role of type I collagen [col(I)], a principal constituent of the ECM in tumour metastases, in RR of RCC is unknown.

Methods. RCC cell adhesion to matrix was studied via precoating a variety of ECM glycoproteins onto plates. Cancer cell apoptosis and cell cycle were evaluated with flow cytometry using annexin V and propidium iodide stains, respectively. Activation of cellular survival signalling was analysed with western blots, and specific molecular inhibitors were correspondingly employed to block signalling. Hypoxia (<1%) was induced via N_2/CO_2 gas flow in a specialized chamber.

Results. While adherence to col(I) enhanced RCC cell proliferation in general, col(I) and fibronectin, but not fibrinogen, could confer specific anti-apoptotic RR to RCC cells. The radioprotective effect of col(I) was maintained during both hypoxia/reoxygenation and normoxia conditions. In contrast to intact col(I), micronized col(I), lacking the natural fibrillar structure, was not radioprotective. The effect of col(I) in RCC cells is mediated via attenuation of apoptosis rather than cell cycle redistribution, involving the PI3 kinase/Akt pathway but not the MAP kinase pathway.

Conclusions. Adherence to col(I) appears to be a relevant environmental cue enhancing RR in RCC cells, Akt dependently. Our results support inhibition of the PI3-kinase/Akt pathway as a radiosensitizing approach.

Keywords: Akt; collagen type I; hypoxia; ionizing radiation; renal cell carcinoma

Introduction

Palliative ionizing radiation is employed for renal cell carcinoma (RCC) metastasis to the bones and brain. However, a small surviving fraction of irradiated cancer cells can potentially generate a growing resistant cell population [1]. Because advanced stage RCC is considered radiation resistant (RR), understanding the molecular mechanisms of RR is of critical significance. RR may derive from constitutive survival signalling via (1) oncogenic mutations, e.g. *Ras*; (2) activation of tyrosine kinase receptors, e.g. *EGFR* or (3) loss or silencing of tumour suppressors, e.g. *PTEN* [2]. In this context, the tumour microenvironment critically influences cancer cell survival, where the extracellular matrix (ECM) protects from several types of apoptosis, i.e. anoikis, growth factor withdrawal, death receptor activation, drug-induced genotoxicity and UV radiation [3–6]. Cell adhesion to matrix elicits integrin-mediated cell protection from both intrinsic and extrinsic apoptotic cascades [4,7–10], induced by cytotoxic drugs [11,12] or death receptor ligands [4,13], respectively. Thus, ECM contributes to drug resistance and failure of standard cancer therapy that may lead to resistant clones [14].

Currently available models for drug resistance include (1) homo- or heterocellular cell–cell interactions in spheroid cell cultures (where adherent cells secrete their own matrix glycoproteins) [15] (2) cell–substrate interaction using mixed stroma (e.g. matrigel), comprising ill-defined matrix glycoproteins and soluble growth factors and (3) cell adhesion to a defined, pre-coated matrix glycoprotein, primarily fibronectin (FN). FN and vitronectin (VN) are commonly considered the active components within the ECM, serving as the main integrin receptor ligands [12,16], and FN coating model is commonly used for cell adherence to ECM [14].

In vitro ECM-mediated resistance has been previously described for leucaemia, myeloma, lung, melanoma, glioma, pancreatic and colon cancer cells [12,14,17–20]

and appears to be mediated primarily via β1 integrins [10]. While cell culture on ECM has been practical for the study of intracellular survival mechanisms eliciting anti-apoptotic signalling [4,13], this is not a universal protective mechanism. Thus, for example, FN protected myeloid leucaemia cells from DNA-damaging agents, e.g. mitoxantrone, and the microtubule inhibitor, docetaxel [21], but not from another microtubule inhibitor, vincristine [11].

ECM has also been studied in the context of radiation resistance (RR). Ionizing radiation results in both DNA breaks and reactive oxygen species (ROS)-induced cell damage. The DNA damage includes single- and double-strand DNA breaks, DNA base damage and DNA–DNA or DNA–protein cross-links [22]. DNA damage results in apoptosis via the release of mitochondrial cytochrome c and activation of the intrinsic apoptotic cascade. Therefore, identification of the basic mechanisms conferring RR in RCC and other tumours may allow the development of apoptosis-enhancing, radiosensitizing agents, thereby decreasing the radiation dose inflicted on neighbouring normal tissues. ECM has been reported to confer RR, where the residual tumour cell niches within the tumour microenvironment surviving radiation may be driven to proliferation and long-term resistance to multiple cytotoxic stresses [16,22,23].

The molecular mechanisms of ECM-RR have recently begun to unfold [11,24]. ECM appears to activate β1 integrin signalling, thereby transducing survival signals following adhesion to FN and collagen type III in murine fibroblasts [24]. ECM-RR has been previously reported in myeloid leucaemia cells adherent to FN [11] and in fibroblasts and human glioma, breast and lung cancer cells [24]. In contrast to FN, type I collagen [col(I)] has been less extensively studied in the context of ECM-RR and its study was limited to haematopoietic cells [16].

The rationale for studying col(I)-ECM-RR in RCC cells derives from reports identifying col(I) expression as a principal signature of metastases [25]. On the basis of the reported role of β1 integrins in drug resistance and RR, ECM ligands such as FN (ligand for α5β1) and col(I) (ligand for α1β1, α2β1 and α3β1) may be optimal to elicit resistance. Col(I) is the most abundant constituent of the ECM found in most connective tissues and primarily in the bone, cornea, tendon and dermis. In the latter, for example, col(I) and col(III) comprise roughly 85% and 10% of all collagen, respectively. Both types of collagen are synthesized as α procollagens [26], of which at least 30 gene copies exist [27]. Col(I) is a triple-stranded helical protein comprising two α1 chains and one α2 chain, forming a fibril structure essential for its function and induction of signalling. Col(I) has a number of roles within the ECM, i.e. a mechanical scaffold, binding of growth factors, regulation of cell cycle, modulation of gene expression and support of angiogenesis [27,28]. In the context of the tumour microenvironment, col(I) is overexpressed in primary and metastatic carcinomas [25,29–31]. Col(I) deposition is also associated with support of cancer cell invasion and neoangiogenesis [31]. Thus, characterization of col-(I)-ECM-RR and its underlying mechanisms may contribute to the understanding of RR in RCC.

Materials and methods

Cells and reagents

The human RCC cell line, CaKi-1, originally derived from RCC metastasis, and the human embryonic kidney (HEK) 293 tubular epithelial cell line were both from ATCC. Mel624 human melanoma cells were from S. Rosenberg (NIH). Other human melanoma cell lines included MO and MO-αIIb (isogenic lines, differing in stable expression of the integrin αIIb, both from Mark Ginsberg, the Scripps Research Institute, La Jolla, CA, USA). All culture media, fetal calf serum and supplements were purchased from Biological Industries (Beit-Haemek, Israel). Low-serum culture (0.5% serum) was employed to eliminate survival signalling induced by growth factors and to highlight the net effect of adhesion to ECM, as previously reported [7,24,32]. The PI3 kinase (PI3K) inhibitor LY294002 [(2-(4-morpholinyl)-8-phenyl-chromone, Sigma] preferred over Wortmannin due to superior stability [33] and the MAP kinase/Erk pathway MKK1 inhibitor PD98059 (Calbiochem, San Diego, CA, USA) were alliquoted to stock solutions in DMSO (Sigma). Working concentration for both inhibitors was 20 μg/ml. Controls for kinase inhibitors included DMSO only. Rat col(I), bearing 92.1% homology to human col(I), was produced as previously reported from rat tail tendons [34] and stored at 4°C until used. Micronized collagen, used in the cosmetics industry as a dermal filler, consists of 95% denatured col(I) and is devoid of its original 3D fibrillar structure [35]. In this study, we used micronized bovine collagen (Ortec, New York, NY, USA), comprising 95% col(I) and 5% col(III), suspended as 3.5% in physiologic PBS solution.

Radiosensitivity assays

To evaluate apoptosis in RCC cells, we used annexin V flow cytometry (see below) rather than clonogenic assays. One million CaKi-1 or control melanoma cells were cultured serum-replete, trypsinized, counted and cultured at 0.5% serum in 12-well plates for 24 h on either uncoated or pre-coated wells with the indicated concentration of col(I), FN or fibrinogen.

After 24-h culture with or without a tyrosine kinase inhibitor (see below) [36,37], the plates were sealed with tape and set on the flat phantom, 1 cm in thickness, to deliver an accurate absorption dose to the cells, and single-fraction irradiated at room temperature using a high-dose rate, radium-operated gamma cell irradiator. Controls were sham irradiated.

Assessment of cell growth and viability

The MTS colorimetric assay (Promega) was used as described before [38]. Briefly, the number of living cells was indirectly measured by optic density (OD) using a plate reader. Pre-calibration of OD readings with the number of seeded cells allowed the quantitative measurement for each cell line. Viability was also measured with flow cytometry analysis (see below) as propidium iodide (PI)-negative and annexin V-negative cells (see below).

Determination of the cell death mechanism

Apoptotic cells were detected by double-staining flow cytometry (Roche Molecular Biochemicals), as described before [39]. Briefly, annexin V was used to probe outer membrane localization of phosphatidyl serine as a marker of apoptosis. Counterstaining was with propidium iodide (PI). Viable cells were defined as PI-negative, annexin V-negative; necrotic cells were stained PI-positive, annexin V-negative; apoptotic cells were PI-negative and annexin V-positive, while PI-positive, annexin V-positive cells were defined as dead of indeterminate cause. Flow cytometry results were analysed with the FCS Express Professional software (www.denovosoftware.com).

Hypoxia induction

Short (6 h) hypoxia (<1% oxygen saturation) was induced using a continuous flow of 95% N_2 and 5% CO_2 in a double-chamber device maintained at 37°C by heated water circulation [40]. Next, cells were either irradiated or sham irradiated, reoxygenated for 48 h and evaluated for viability and apoptosis.

Cell cycle analysis

Caki-1 cells were grown for 24 h on either uncoated plates or pre-coated with col(I). The nuclear DNA was stained with PI, plotted against the nuclear number and presented for each cell cycle phase as a percentage of the total number of cells [38].

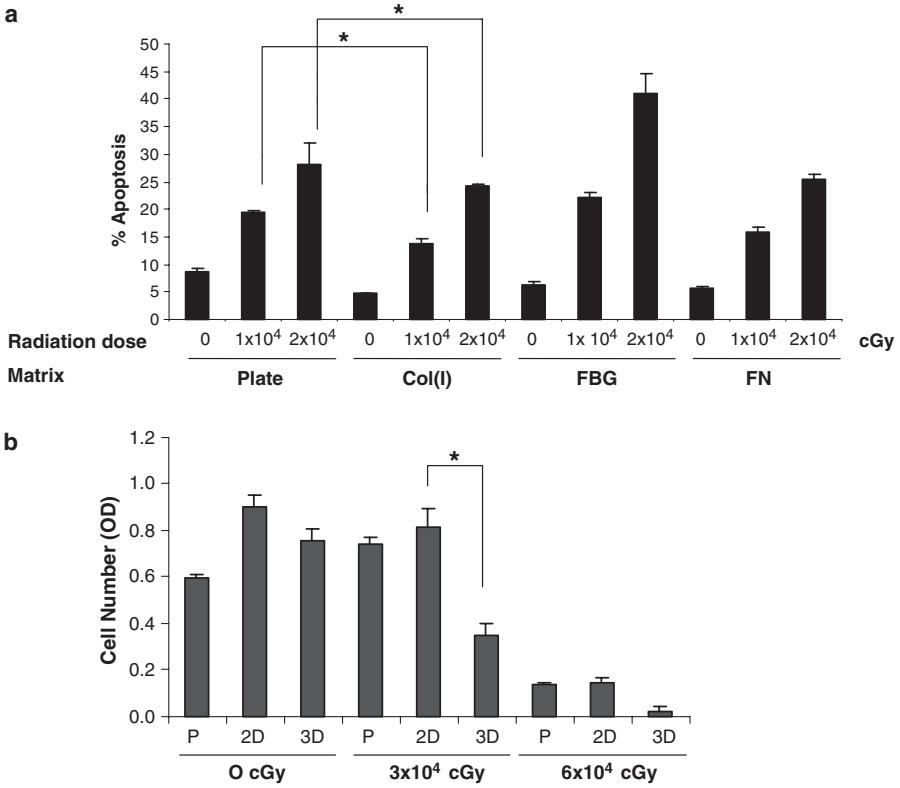

Fig. 1. Adhesion to col(I) confers radiation resistance (RR) to renal cancer cells. (**a**) CaKi-1 cells were cultured for 24 h at 0.5% serum on plates coated with the indicated matrix glycoprotein, irradiated with the indicated dose, cultured for another 48 h and analysed for apoptosis with annexin V/PI flow cytometry. FN, fibronectin; Col(I), type I collagen; FBG, fibrinogen. ∗, $P < 0.05$ for decreased apoptosis in col(I)-adherent cells. (**b**) An intact fibrillar structure of col(I) is required to elicit RR. CaKi-1 cells were cultured on 2D plates pre-coated with fibrillar intact col(I) (2D), on 3D micronized (denatured) col(I) in suspension (3D) or on uncoated plates. Cells were either sham-irradiated or irradiated at the indicated doses and the number of viable cells was measured 48 h after irradiation with the MTS assay. ∗, $P < 0.05$ for radioresistance in cells adherent to intact versus denatured col(I).

Western blot assays

Whole cell lysates were prepared via lysis in SDS buffer and sonication, resuspension in PBS, centrifugation and lysis for 1 h on ice using a lysis buffer containing a complete protease inhibitor cocktail (Roche Applied Science). The concentration of protein extracts was measured using the Bradford assay. The samples were boiled, sheared and stored at −20°C. The total protein extract (20 μg) was separated on a 12% SDS gel and blotted onto the nitrocellulose membrane and blocked by 3% nonfat milk. To detect the indicated kinase, we incubated it with the primary antibody diluted at 1:1000 in tris-buffered saline with 0.01% Tween 20. The primary antibodies used were anti-Erk and anti-p-Erk (Cell Signaling Technology, Danvers, MA, USA), anti-Akt and anti-p-Akt, anti-Fak and anti-p-Fak (from Santa Cruz, CA) and anti-GAPDH (Cell Signaling Technology). Next, the membrane was reprobed with a peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ, USA) and chemiluminescence was used for signal detection.

Statistical method

All experiments were performed twice in triplicates and results were analysed with two-tailed *t*-test. A $P < 0.05$ was considered statistically significant.

Results

Determination of IR LD50 in RCCs

A dose-response radiosensitivity curve was prepared to identify the lethal dose 50 (LD50) required to reduce viability by 50% in HEK 293, human RCC (CaKi-1) and human melanoma cell lines (Supplementary Figure 1). Low dose radiation shifted serum-depleted HEK293 cells to apoptosis. However, when cultured with full serum, the radiation sensitivity of HEK293 cells was similar to serum-depleted CaKi-1 cells, suggesting ligand-induced radioprotection. The LD50 identified for each cell line was used for subsequent experiments.

Adherence to col(I) protects RCC cells from radiation

To evaluate the effect of various ECM glycoproteins on radiation-induced apoptosis, CaKi-1 RCC cells were cultured serum-depleted on uncoated plates or on plates pre-coated with either FN, col(I), and fibrinogen. Serum depletion was employed as described before [7,32] to directly study ECM-derived survival signalling, independently of activation by growth factors.

Col(I) and FN conferred a similar degree of protective anti-apoptotic ECM-RR, while fibrinogen enhanced radiation-induced apoptosis (Figure 1a). Col(I)-ECM provided specific radioprotection as it did not protect from another insult, e.g. viral oncolysis (not shown).

We further observed that col(I)-ECM-RR depends on the natural conformation of col(I) (Figure 1b). While CaKi-1 cells could proliferate well on micronized, denatured col(I), only intact col(I,) but not denatured col(I) ECM, could confer radioprotection (Figure 1b). Thus, ECM-RR is not

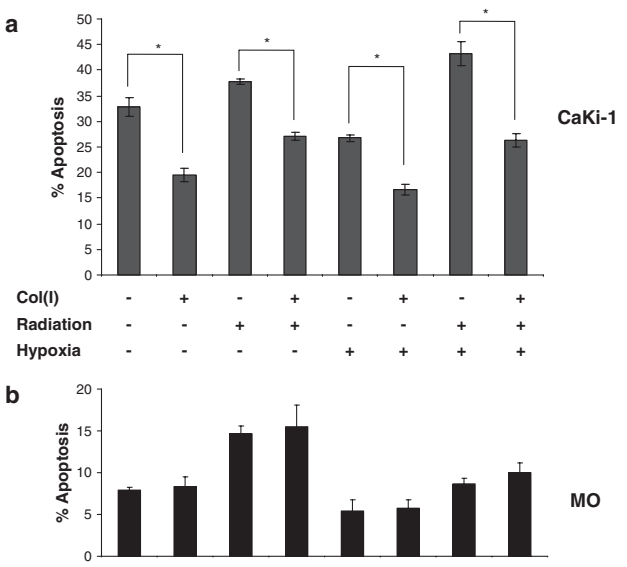

Fig. 2. The radioprotective effect of col(I) is maintained during acute hypoxia/reoxygenation. CaKi-1 renal cancer cells (**a**) or MO melanoma cells (**b**) were cultured at 0.5% serum on col(I)-coated or on uncoated plates for 24 h. Cells were then either incubated for 6 h in a hypoxic chamber (see the Material and Method section) or at normoxia at 37°C. Cells were reoxygenated at normoxic conditions for 30 min and either irradiated or sham irradiated. Cell culture was then resumed for another 48 h and analysed by flow cytometry for apoptosis (see the Material and Method section). ∗, $P < 0.05$ for decreased apoptosis in col(I)-adherent cells.

mediated by adhesion *per se* but appears to require signalling specifically elicited by col(I) (and FN).

Because FN, unlike col(I), has already been extensively studied in this context and because col(I) deposition is a hallmark of tumour metastases [25], we next focused on col(I)-ECM-RR in CaKi-1 RCC cells.

Col(I)-ECM-RR is maintained during hypoxia

Acute hypoxia (6 h) was reported to confer RR to cancer cells in contrast to chronic hypoxia (72 h) [41]. We thus evaluated whether the radioprotective effect of col(I) is maintained in the setting of acute hypoxia/reoxygenation (Figure 2). In these experiments, we compared CaKi-1 cells, known to display a variety of integrin receptors [42], to the melanoma MO cells, specifically selected for integrin receptor downregulation [43]. While in CaKi-1 col(I)-ECM-RR was maintained in all cell culture conditions tested (Figure 2a), MO cells were indifferent to col(I) (Figure 2b). Of note, while acute hypoxia radiosensitized CaKi-1 cells, it attenuated apoptosis in non-irradiated CaKi-1 cells (Figure 2a). Taken together, col(I)-ECM-RR is maintained in CaKi-1 cells over a range of apoptotic insults relevant to ischaemic RCC tumours, i.e. growth-factor withdrawal, hypoxia/reoxygenation and ionizing radiation (Figure 2a).

The mechanism of Col(I)-CAM-RR in RCC cells involves attenuation of apoptosis

Cell proliferation *per se* could not account for CAM-RR because CaKi-1 cells efficiently growing on fibrinogen

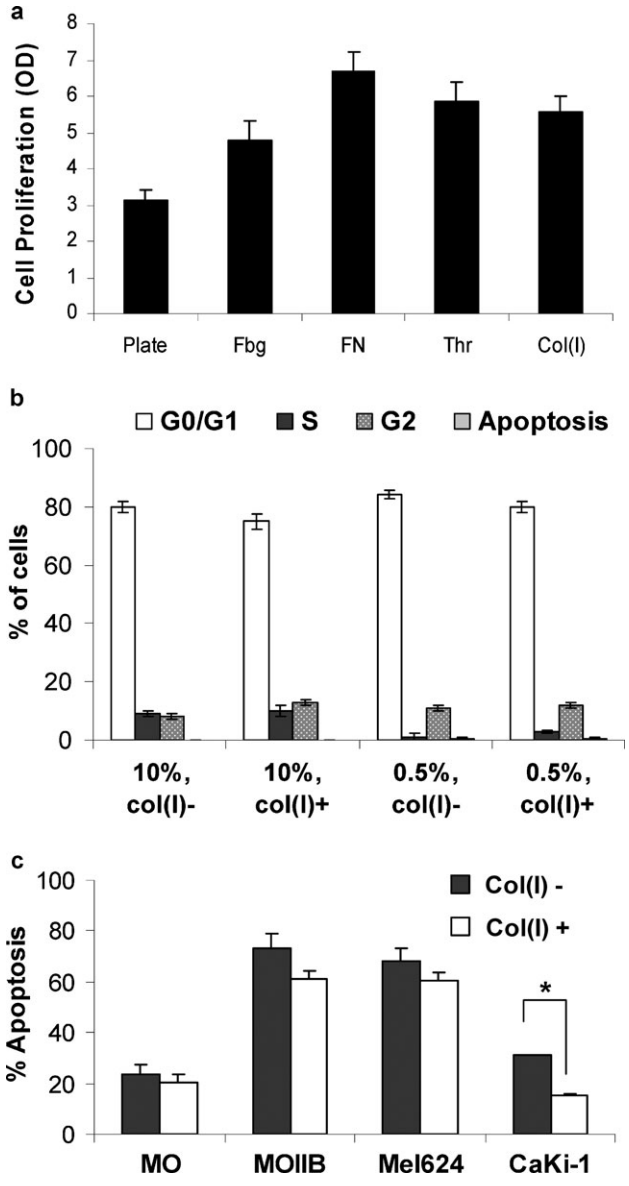

Fig. 3. The protective mechanism of col(I) derives from attenuation of apoptosis. (**a**) Proliferation of non-irradiated CaKi-1 cells on the indicated matrix proteins was measured after 48 h culture at 0.5% serum using the MTS assay. ∗, $P < 0.05$. Fbg, fibrinogen; FN, fibronectin; Thr, thrombin; Col(I), collagen type I. (**b**) CaKi-1 renal cancer cells were cultured at 0.5% or 10% serum on col(I)-coated or on uncoated plates for 24 h and analysed for the cell cycle phase using nuclear DNA staining with propidium iodide (PI). The percentage of cell number in each cell cycle phase is plotted in the *y*-axis. Cells at SubG1 are marked as apoptosis. (**c**) An anti-apoptotic effect of adhesion to col(I), independent of radiation, was evaluated in CaKi-1 and the human melanoma cell lines MO, MOIIB and Mel624. Apoptosis was induced by a prolonged, 72 h complete serum-depletion and measured as above by annexin V flow cytometry. ∗, $P < 0.05$ for decreased apoptosis in CaKi-1 adherent to col(I) versus plate.

(Figure 3a) or micronized col(I) (Figure 1b) were prone to radiation-induced apoptosis. Previously, ECM-RR was reported to involve either cell cycle arrest or activation of anti-apoptotic signalling [44,45]. While serum-depletion alone induced both G_0/G_1 and G_2 cell cycle arrest, cell adherence to col(I) induced cell cycle progression from G_0/G_1 to S and G_2, facilitated by serum repletion (Figure 3b).

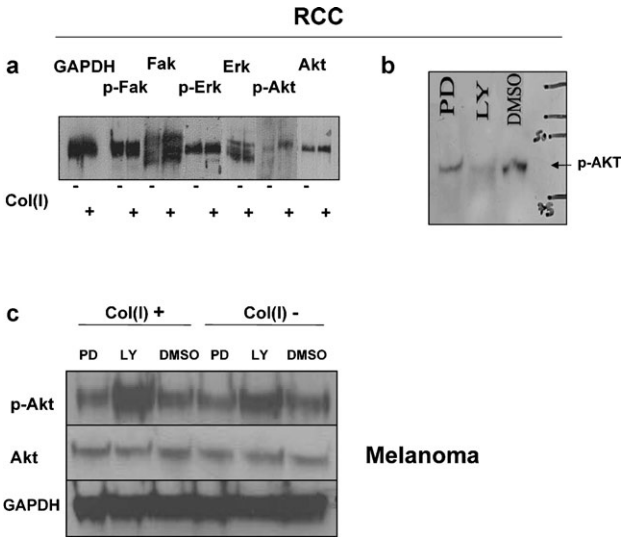

Fig. 4. Col(I) enhances Akt phosphorylation in CaKi-1 cells, while in Mel624 melanoma cells Akt is constitutively phosphorylated. Caki-1 RCC cells and Mel624 melanoma cells were cultured at 0.5% serum on col(I)-coated or on uncoated plates for 24 h. Cells were harvested, protein extracted and Western blotted with antibodies recognizing total Fak, Erk and Akt, and with antibodies specific for their phosphorylated counterparts. Loading control was GAPDH. (**a**) The effect of adherence to col(I) on kinase activation in CaKi-1 cells. (**b**) CaKi-1 cells were cultured on col(I) and incubated with 20 μM of LY290042, PD95089 or DMSO vehicle for 24 h and blotted for Akt phosphorylation. (**c**) Mel624 melanoma cells were cultured with or without adherence to col(I) and with or without the PI3 kinase/Akt inhibitor LY294002 or the MEK inhibitor PD95089 or DMSO for 24 h, followed by protein extraction and western blotting for Akt phosphorylation.

Because cells are most radiosensitive in the G_2/M phase and less sensitive in the G_1 phase [46,47], col(I)-induced ECM-RR in CaKi-1 cells does not appear to result from the cell cycle redistribution it inflicts.

In contrast, we found that adherence to col(I) efficiently attenuated apoptosis in RCC CaKi-1 cells, such as induced by prolonged complete serum depletion (Figure 3c) or radiation (Figure 1). Thus, col(I) effect in CaKi-1 cells is mediated, at least partially, by attenuation of apoptosis.

Col(I) effect in RCC cells is Akt dependent

To evaluate activation of survival signalling accounting for the anti-apoptotic effect elicited by col(I), we next performed Western blots for key effectors of integrin ligation [5,8], e.g. focal adhesion kinase (Fak) or the downstream effectors of Ras, i.e. the phosphatidylinositol-3 kinase (PI3K)/Akt and MAP kinase/Erk pathways.

We found that Akt phosphorylation, but not Erk or Fak phosphorylation, was induced in CaKi-1 cells by adherence to col(I) (Figure 4a). In control Mel624 melanoma cells, Akt was constitutively phosphorylated independently of col(I) (Figure 4b). In accord with these findings, the PI3 kinase inhibitor LY294002 efficiently decreased Akt phosphorylation in RCC CaKi-1 cells (Figure 4c), but not in melanoma Mel624 cells (Figure 4b). Consequently, the PI3K inhibitor LY294002, but not the MKK1 (of the MAP kinase pathway) inhibitor, PD98059, could efficiently kill CaKi-1 cells (Supplementary Figure 2A) via apoptosis (Supplementary Figures 2B and 5a).

Next, we evaluated whether Akt inhibition using LY294002 could mitigate the radioprotective effect of adherence to col(I). LY294002 pre-radiation incubation could efficiently mitigate col(I)-ECM-RR, enhancing apoptosis from 10.8% to 40.63% (Figure 5a). However, when CaKi-1 cells were treated with LY290042 post-radiation, its radiosensitizing and col(I)-mitigating effects were maintained, yet less efficient than pre-irradiation incubation (Figure 5b). Thus, Akt inhibition radiosensitizes CaKi-1 cells and can mitigate, at least partially, the radioprotective effect of col(I).

Discussion

Ionizing radiation is employed for RCC metastasis as a palliative measure. While RCC is considered inherently RR, the underlying mechanisms are unknown. The emerging finding of ECM-RR may partially account for the failure of clinical trials to recapitulate efficient radiosenstization observed *in vitro* using blockade of growth factor receptors, i.e. ERBB or EGFR [48], possibly via ECM-oncogene activation downstream of growth factor receptors [22,35]. Because this effect is not limited to the first course of radiation therapy [11], attenuation of apoptotic damage may subsequently result in increased genetic instability leading to emergence of new resistant cancer clones [23]. Thus, understanding the molecular mechanisms of ECM-RR may allow better radiosensitization protocols for metastatic tumours inclusive of RCC metastasis.

Factors accounting for RR may include (1) G1 cell cycle arrest (2) enhanced DNA repair in the S phase of the cell cycle (3) intratumoural hypoxia and (4) augmented anti-apoptotic signalling [44,45]. In this *in vitro* study, we evaluated some of these four possible mechanisms in RCC cells adherent to col(I).

First, the cell cycle redistribution induced by adherence to col(I) cannot account for RR because adherence to col(I) shifted cells from G_1 to G_2 (Figure 3a). In contrast, radiosensitivity decreases in the G_1 phase [45] and even more prominently during the latter S phase where DNA damage repair is maximal, while cells are most radiosensitive in the G_2/M phase when DNA repair enzymes are degraded.

Secondly, DNA repair by the ataxia telangiectasia mutated (ATM), ATR (ATM and Rad3-related kinase) and DNA–protein kinase (DNA–PK) is typically constitutively active in cancer cells and may further be induced by radiation itself [22]. ATM and ATR-dependent cell cycle checkpoints may cause cell cycle arrest and repair of ROS-mediated DNA damage.

While we did not directly evaluate the effect of col(I) on DNA repair activity, known off targets of LY294002 may include DNA–PK and ATM [49], thereby possibly also accounting for some of its potent radiosensitizing effect.

Thirdly, acute hypoxia may confer RR either via decreased degradation of the hypoxia inducible factor 1α (HIF1α) [50], through enhanced DNA repair by ATM and ATR or via repair of ROS-mediated DNA damage. A combined effect of acute hypoxia/reoxygenation on

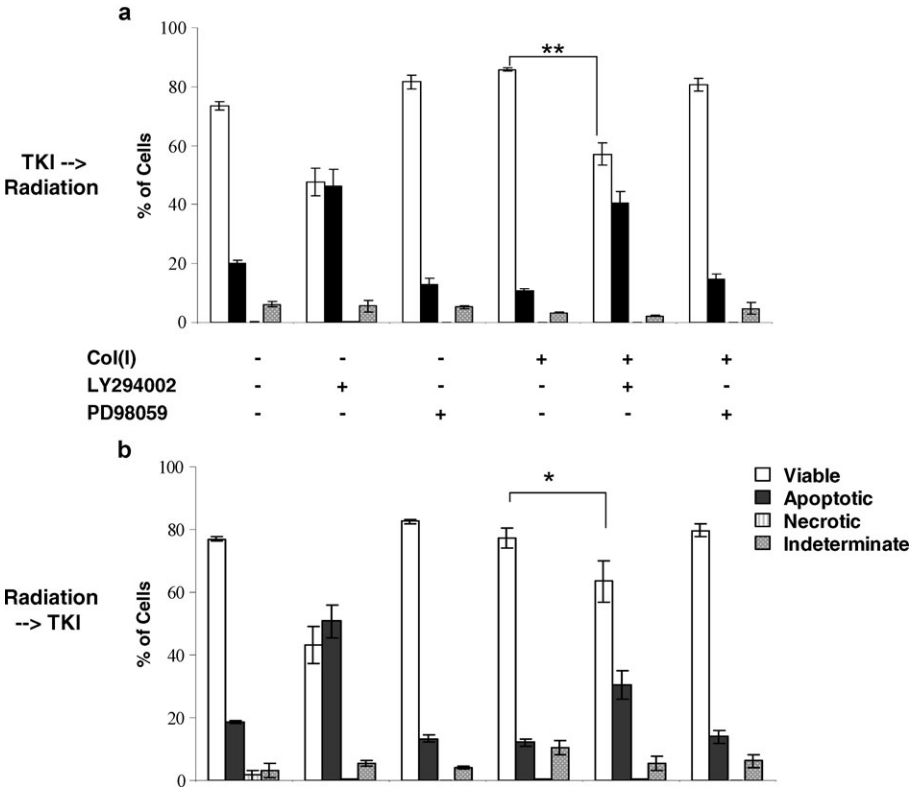

Fig. 5. PI3 kinase/Akt inhibition mitigates the radioprotective effect of col(I). Caki-1 cells were cultured serum-depleted on uncoated plates or pre-coated with col(I) for 24 h and irradiated with 20 000 cGy. Cells were incubated with the tyrosine kinase inhibitors (TKI) LY290042, PD98059 or DMSO vehicle alone either 24 h before irradiation (**a**) or immediately after irradiation (**b**). Forty-eight hours after irradiation cells were analysed by annexin v/PI flow cytometry. $**$, $P < 0.01$; $*$, $P < 0.05$ for LY294002-induced decreased cell viability in cells adherent to col(I).

ECM-RR was not evaluated before in any cancer cell line, to the best of our knowledge. In CaKi-1 cells, acute hypoxia/reoxygenation had a radiosensitizing effect that was mitigated by adherence to col(I) (Figure 2a). Possible mechanisms accounting for radiosensitization by hypoxia/reoxygenation may involve destabilization of HIF1α, generation of ROS and cell cycle progression [51]. Hypoxic tumours often reoxygenate after fractionated radiation, probably due to reduction in oxygen consumption following death of the radiosensitive tumour cells.

The capacity of CaKi-1 cell adherence to col(I) to inhibit the hypoxic radiosensitizing effect in CaKi-1 (Figure 2a) may indicate downstream activation of anti-apoptotic cascades (Figure 3) or DNA-repair mechanisms. In contrast, in MO melanoma cells, previously selected for integrin downregulation, adherence to col(I) did not confer RR (Figure 2b). Thus, the radioprotective effect in RCC cells adhering to col(I) under a variety of culture conditions mimicking intratumoural conditions appears to involve integrin-mediated anti-apoptotic signalling.

Fourthly, our studies suggest a critical role of PI3K/Akt survival signalling mediating RR in RCC cells adhering to col(I) (Figures 4 and 5). This finding is in accord with a previous report where overexpression of a constitutively active Akt could restore RR despite use of LY294002 [36]. While PI3K/Akt inhibition may radiosensitize cancer cells in general [36,37,52], we suggest that this approach may be specifically relevant to circumvent col(I)-ECM-RR in RCC cells.

Of note, β1 integrins, receptors for col(I) and FN were previously shown to confer cancer cell resistance to chemotherapy and radiation via formation of inhibitory complexes of p-Akt with procaspase 8 [16].

In conclusion, cancer cell response to radiation depends to a large extent on the coordinated signalling pathways controlled by cellular communication with the ECM. In this *in vitro* study, we highlight the role of col(I)-ECM-RR in RCC cells. An extrapolation of our *in vitro* findings may suggest that to target specific niches within RCC metastasis where cancer cells may be in direct contact with col(I), Akt inhibition may be required. The setbacks of our study may include (1) *in vitro* immobilization of col(I), possibly exposing cryptic epitopes that may support invasive phenotypes and (2) the lack of fractionated radiation. Nevertheless, our data support an integrative radiosensitization approach comprising pre-radiation administration of PI3K/Akt inhibitors to alleviate ECM-mediated radiation resistance of RCC cells.

Acknowledgements. This study was supported by the German-Israeli Foundation (grant no 817/2004 to YSH, RG & DMN), the Israel Science Foundation grant no. 573/03 (YSH) and the Israeli Ministry of Health, Chief Scientist Office (YSH).

Conflict of interest statement. None declared.

Supplementary data

Supplementary data is available online at http://ndt.oxfordjournals.org.

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Received for publication: 29.12.08; Accepted in revised form: 11.9.09

Nephrol Dial Transplant (2010) 25: 380–388
doi: 10.1093/ndt/gfp505
Advance Access publication 30 September 2009

Expression of renin–angiotensin system signalling compounds in maternal protein-restricted rats: effect on renal sodium excretion and blood pressure

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Abstract

Background. Intrauterine growth restriction due to low maternal dietary protein during pregnancy is associated with retardation of foetal growth, renal alterations and adult hypertension. The renin–angiotensin system (RAS) is a coordinated hormonal cascade in the control of cardiovascular, renal and adrenal function that governs body fluid and electrolyte balance, as well as arterial pressure. In the kidney, all the components of the renin–angiotensin system including angiotensin II type 1 (AT1) and type 2 (AT2) receptors are expressed locally during nephrogenesis. Hence, we investigated whether low protein diet intake during pregnancy altered kidney and adrenal expression of $AT1_R$ and $AT2_R$ receptors, their pathways and if the modified expression of the RAS compounds occurs associated with changes in urinary sodium and in arterial blood pressure in sixteen-week-old males' offspring of the underfed group.

Methods. The pregnancy dams were divided in two groups: with normal protein diet (pups named NP) (17% protein) or low protein diet (pups LP) (6% protein) during all pregnancy.

Results. The present data confirm a significant enhancement in arterial pressure in the LP group. Furthermore, the study showed a significantly decreased expression of RAS pathway protein and Ang II receptors in the kidney and an increased expression in the adrenal of LP rats. The detailed immunohistochemical analysis of RAS signalling proteins in the kidney confirm the immunoblotting results for both groups. The present investigation also showed a pronounced decrease in fractional urinary sodium excretion in maternal protein-restricted offspring, compared with the NP age-matched group. This occurred despite unchanged creatinine clearance.

Conclusions. The current data led us to hypothesize that foetal undernutrition could be associated with decreased kidney expression of AT_R resulting in the inability of renal tubules to handle the hydro-electrolyte balance, consequently causing arterial hypertension.

Keywords: angiotensin receptors; hypertension; low protein diet; renal function; renin–angiotensin system