

**REVIEW**

Urinary bladder carcinogenesis induced by chronic exposure to persistent low-dose ionizing radiation after Chernobyl accident

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Urinary bladder urothelium as well as cells in the microenvironment of lamina propria (endothelial elements, fibroblasts and lymphocytes) demonstrate a number of responses to chronic persistent long-term, low-dose ionizing radiation (IR). Thus, oxidative stress occurs, accompanied by up-regulation of at least two signaling pathways (P38 mitogen-activated protein kinase and nuclear factor-kB cascades) and activation of growth factor receptors, in the bladder urothelium of people living in Cesium 137-contaminated areas of Ukraine, resulting in chronic inflammation and the development of proliferative atypical cystitis, so-called Chernobyl cystitis, which is considered a possible pre-neoplastic condition in humans. Furthermore, significant alterations in regulation of cell cycle transitions are associated with increased cell proliferation, along with up-regulated ubiquitination and sumoylation processes as well as inefficient DNA repair (base and nucleotide excision repair pathways) in the affected urothelium. The microenvironmental changes induced by chronic long-term, low-dose IR also appear to promote angiogenesis and remodeling of the extracellular matrix that could facilitate invasion as well as progression of pre-existing initiated cells to malignancy. Based on the available findings, new strategies have been developed for predicting and treatment of Chernobyl cystitis—a first step in urinary bladder carcinogenesis in humans.

**Introduction**

The Institute of Urology (Academy of Medical Sciences of Ukraine) in Kiev during 1994–2006 collected all benign prostate hypertrophy (BPH) patients who underwent suprapubic prostatectomy, and all these patients were included in our study in different years without exception, along with a small number of females with chronic cystitis. A computerized register of the patients enrolled from 1994 until now has been established (totally 592 patients: males 559 and females 33). In this paper, an overview is given on the biological effects of chronic long-term and persistent low-dose ionizing radiation (IR) on urinary bladder urothelium of people continuously living in Cesium 137 (137Cs)-contaminated areas of Ukraine, where ecological problems still remain after some 20 years. A series of histological, immunohistochemical and molecular biological investigations were performed for this purpose.

**Chernobyl cystitis**

We have documented for the first time that chronic long-term, low-dose IR in parts of the Ukraine leads to development of a previously unknown urinary bladder disease, radiation-induced chronic proliferative atypical cystitis or so-called Chernobyl cystitis (1). This is characterized by multiple areas of dysplasia and carcinoma in situ (CIS) of the urinary bladder urothelium in strong association with sclerosis of connective tissue and strongly increased angiogenesis without a marked inflammatory reaction in the propria mucosa.

The incidences of urinary bladder dysplasias and carcinomas in 164 patients living in 5–30 Ci/km² (group 1), 0.5–5 Ci/km² (group 2) soil contamination areas or non-contaminated areas (group 3) are summarized in Table I. Urinary bladder epithelium biopsied from 131 male patients with BPH from groups 1 and 2 demonstrated multiple areas of dysplasia with strong epithelial abnormalities such as extensive cellular pleomorphism and nuclear hyperchromatism, frequently combined with increased thickness of urothelial epithelium. Foci of dysplasia were observed in 97 and 83% of cases in groups 1 and 2, respectively, and multiple areas of CIS with neoplastic changes of urothelium that frequently involved von Brunn’s nests or cystitis cystica were detected in 73 and 64%. Nine small papillary or invasive transitional cell carcinomas (pTa–pT1) were also incidentally found in individuals from radio-contaminated areas.

Additionally, all cases in groups 1 and 2 exhibited proliferative cystitis, i.e. von Brunn’s nests, cystitis cystica and squamous and glandular metaplasia, which were frequently combined and had marked features of chronic radiation cystitis rather than simple inflammation. Large areas of sclerosis of connective tissue in lamina propria with less prominent inflammatory cellular infiltration of lymphocytes, histiocytes and plasma cells were typical. Among these lesions, definite new vascularization, sometimes with the development of angiomatoid-like vessels full of erythrocytes and hemorrhage, was detected in 62, 53 and 6% of groups 1, 2 and 3, respectively. Endothelial proliferation of new micro-vessels in lamina propria was markedly increased in 46 and 34% of cases in groups 1 and 2, respectively. In all female patients, large areas of sclerosis of connective tissue with some inflammatory infiltration and large areas of hyper-vascularization and patchy hemorrhage were detected in lamina propria (Figure 1). Patterns of chronic cystitis without proliferative changes but with clear cell urothelium and marked inflammatory cellular infiltration in lamina propria were found in patients of group 3. However, hypervascularization and development of angiomatoid-like vessels were not observed.

Unfortunately, we have no means to determine what radiation dose our patients received. However, we obtained results of 137Cs measurement in 1 day urine of the same patients with BPH in groups 1 and 2; the values are summarized in Table II. Significant elevation of 137Cs levels was found in patients from group 1 (6.47 Bq/l) and to a lesser extent in patients from group 2 (1.23 Bq/l), as compared with group 3 patients (0.29 Bq/l). Our radiometric study showed significant increase of 137Cs in urine of patients from groups 1 and 2, who suffered from BPH and presumably urinary retention, so that radiation exposure of the urothelium would be expected to have been high. Such male patients with BPH could be the group with the highest risk of Chernobyl cystitis. Our female patients with symptoms of chronic cystitis from groups 1 and 2, which were without urinary retention...
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Cell cycle regulation. Cell cycle check points function to maintain genetic stability by providing additional time for repair of DNA damage and completion of events that are necessary for accurate cell division. Some check points, such as the DNA damage G1 check point, are dependent on p53, whereas others, such as the decatenation G2 check point, are not (4). The molecular, genetic and cellular changes that occur in urothelial carcinomas are numerous and include loss of cell growth regulation. Several genetic alterations in bladder cancer have been linked with inactivation of tumor-suppressor genes (4). In bladder urothelial carcinomas, one of the most frequently inactivated tumor-suppressor genes is the p53 gene. Genetic alterations in p53 occur in ~50% of bladder carcinomas and are associated with high-stage and high-grade urothelial lesions (5). Spruck et al. have suggested the participation of two molecular pathways in urinary bladder carcinogenesis, with p53 alterations occurring early in CIS and dysplasia before the development of non-papillary invasive lesions but occurring late in papillary urothelial carcinomas (6). Thus, early detection of p53 mutations in urinary bladder epithelial lesions (Chernobyl cystitis) may be strongly predictive of future urinary bladder cancer, especially of the non-papillary invasive type (7).

Our pilot studies indicated increased proliferative activity and possible genetic instability, with over-expression of p53, proliferating cell nuclear antigen (PCNA) and mdm2 in the bladder urothelium of exposed individuals with Chernobyl cystitis (8). The staining intensities of p53, PCNA, p21WAF1/Cip1 and cyclin D1 were directly correlated with levels of radiation exposure and amounts of 137Cs excreted in the urine by patients of groups 1 and 2. The patients from ‘clean’ (not radio contaminated) areas of Ukraine had significantly lower values for all parameters investigated (9).

It has been shown that wild-type p53 plays an essential role in transcription-coupled repair of DNA damage induced by oxidative stress, IR and some chemical carcinogens (10). The p53 protein expression in nuclei of bladder urothelium in both radio-contaminated groups 1 and 2 thus likely represents wild-type p53 accumulation in response to DNA damage. This speculation is supported by aberrant increases of PCNA, cyclin D1 and p21WAF1/Cip1 in the same areas of urothelium, which could be explained by possible involvement of PCNA, cyclin D1 and p21WAF1/Cip1 not only in DNA synthesis but also in DNA excision repair (11). An alternative explanation for the strong p53 protein expression observed in urothelium with Chernobyl cystitis is that it reflects mutations within p53 gene.

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Biopsied urinary bladder specimens were therefore analyzed for mutational inactivation of the p53 gene by polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP) analysis. In addition, urinary sediments collected over intervals were examined by PCR-SSCP and assays of the ability of human p53 to activate transcription in yeast (7). Because human p53 cDNA polymerase chain reaction products can be cloned directly into the reporter yeast strain by homologous recombination without intermediate bacterial cloning steps, the percentage of positive red yeast colonies accurately reflects the mutant p53 mRNA content of the starting material. Therefore, the assay can detect mutant p53 in a minor fraction of cell clones such as those in urine sediments. Mutational analysis of the p53 gene in DNA extracted from the urothelium of patients living in radio-contaminated areas of Ukraine revealed 9 of 17 cases (53%) to harbor one or more mutations within identical or separated samples (7). This frequency is similar to values described for human high-grade, invasive urinary bladder cancers (6). Although base deletions or insertions of the p53 gene have been found in a certain proportion of human urinary bladder carcinomas (12), all p53 mutations identified in this study were single-base-pair substitutions. The most striking feature was the predominance of G:C to A:T transition mutations at CpG dinucleotides, especially in codons 158, 245 and 248. Although IR has been reported to cause a variety of types of DNA damage including single- and double-strand breaks and cross-linking (13), direct in vivo evidence of radiation-induced base-pair substitutions is lacking. Thus, the underlying mechanism might be different from that responsible for the specific mutations observed in this study. In human urinary bladder carcinomas, no specific substitution pattern for the p53 gene has hitherto been described, and there has been no pointer to any specific mutagen (12). On the other hand, mutational analysis of schistosomal urinary bladder carcinomas (endemic in Egypt) gave results that are consistent with our findings; namely, a high proportion of base-pair changes at CpG dinucleotides (18 of 34; 53%) (14). Chronic urinary infection with *Schistosoma hematoobium* is a significant etiological factor in schistosomal urinary bladder carcinomas. Chernobyl cystitis was a common characteristic feature of cases in the present study. Recently, a close relationship between chronic infection and cancer risk has been suggested, with the production of nitric oxide (NO) during inflammatory processes playing a role (15). It has been shown that NO can produce transitions at CpG dinucleotides by deamination of 5-methylcytosine (15). In addition, endogenous formation of urinary N-nitrosocompounds leads to O6-alkylguanine formation and G:C to A:T transitions (14).

Two patients of nine with p53 mutations proved to have multiple p53 mutations in the urinary bladder, in line with the report by Spruck et al. (6) and Goto et al. (16). Different p53 mutations in independent urothelial lesions (case 16) or in metachronous samples (case 17) indicate that a strong carcinogenic insult may have resulted in multiple transformation events in a large field of urothelium, as demonstrated previously in an animal model (17). The frequent (9 of 17 cases, 53%) p53 mutations of altered urinary bladder epithelium in patients with Chernobyl cystitis suggest that prediction of induction of urinary bladder carcinoma may be possible.

Concerning the cell cycle transition regulation, it is necessary to note that activation of cyclin D1 and p21WAF1/Cip1 occurs in response to activation of wild-type p53. It can be speculated that long-term exposure to low doses of 137Cs induces chronic DNA damage in bladder urothelium. An increased level of p21WAF1/Cip1 could thus be interpreted as a manifestation of cellular differentiation. p21WAF1/Cip1 directly interacts with PCNA but the majority of studies show that it does not inhibit PCNA-dependent nucleotide excision repair (11). This suggests that p21WAF1/Cip1 not only plays a critical role in negatively controlling proliferation but also could be activated in a p53-independent manner. About 75% of our cases in both groups 1 and 2 were cyclin D1 positive. It is possible that some of the observed cyclin D1 over-expression occurred in response to activation of wild-type p53 during radiation-induced DNA damage. The dramatic increase of PCNA staining in 88% of cases in groups 1 and 2 involving not only basal cells but also the suprabasal and superficial layers of bladder urothelium is indicative of increased cell proliferation.

In conclusion, the present finding of significant increases in p53, PCNA, cyclin D1 and to a lesser extent p21WAF1/Cip1 expression in the majority of patients with Chernobyl cystitis in groups 1 and 2 chronically exposed to ionizing irradiation, associated with multiple p53 gene transition mutations at CpG dinucleotides, is suggestive of a deregulated cell cycle transition that leads to increased chance of malignant transformation with the possible development of preferentially high-stage and high-grade bladder carcinoma among the population in Ukraine.

**Increased oxidative stress, signaling cascades**

Cellular stress is known to be a multifaceted condition generated by free-radical species, hypoxia and other factors that target the transcriptional factor nuclear factor-κB (NF-κB) with its two major subunit polymorphisms, p50 and p65, and modulate their activity directly or indirectly by activating other signaling cascades (18). The p38 mitogen-activated protein kinase (MAPK) pathway is also involved in primary signaling activated by inducers of stress, such as IR inflammatory cytokines, mediating inhibition of cell proliferation and causing cell death (19). p50/p65 are the active transactivating species of NF-κB, responsible for induction of several genes involved in the control of cell proliferation, apoptosis and, hence, oncogenesis (20).

The pattern of Chernobyl cystitis, including four small incidentally detected pTa tumors in male patients with BPH of the radio-contaminated groups 1 and 2, was associated with strong p53 staining of the urothelial nuclei in the majority of cases (CIS and papillary urothelial carcinomas with severe dysplasia), overlapping with over-expression of the oxidative DNA damage marker, 8-hydroxydeoxyguanosine (8-OHdG), accompanied by elevation of cytoplasmic cyclooxygenase 2 (COX2) and inducible NO synthase (iNOS) (Figure 2). Therefore, our data suggest that the chronic low-dose irradiation could have at least two effects on the urinary bladder urothelium: (i) direct induction of oxidative stress due to irradiation DNA damage as IR is known to be a pure source of reactive oxidative species (21) and (ii) indirect actions with possible consequences for homeostasis and reactions, involving inflammation following enhanced NO production, which triggers a cascade of oncogenic events, possibly initiated by p53 mutations at sites involving CpG dinucleotides (7).

It is necessary to note that chronic infection and inflammation have become well recognized as risk factors for a variety of human cancers. It has been proposed that reactive oxygen and nitrogen species, both formed in infected and inflamed tissues, play roles in the multi-stage carcinogenic process. NO, a potential toxic substance with free-radical properties, is generated from L-arginine by constitutive NO synthase or iNOS. One of the possible mechanisms underlying enhancement of initiation and promotion in carcinogenesis by oxidative stress is the ability to induce prostaglandin formation catalyzed by the enzyme Cox, which converts arachidonic acid to prostaglandins. Presently two Cox enzymes are known Cox1 and Cox2, an important mediators of inflammation, whose synthesis can be stimulated rapidly by tumor promoters (22).

The marked increase of 8-OHdG, COX2 and iNOS expression found in the majority of urothelium samples in groups 1 and 2 is indicative of oxidative stress in its different manifestations. Murata et al. (23) demonstrated that NO induces mutations that inactivate p53 by causing deamination of cytosine, leading to G:C to A:T transitions in vitro. Our data, showing strong 8-OHdG, COX2 and iNOS expression associated with p53 mutational inactivation with frequent G:C to A:T transitions at CpG dinucleotides (in 73% of all mutations) with a hot spot in codon 245 in bladder urothelium of Ukrainian male patients with BPH (7), support the hypothesis that NO-mediated DNA deamination was induced by oxidative stress in groups 1 and 2 bladder urothelium.
Over-expression of p38 MAPK, p65 and p50 subunits of NF-κB by the same cells of urothelium in patients living in the radio-contaminated areas also strongly indicates a pivotal role of oxidative stress. Thus, our results point to low-dose radiation exposure acting on at least two distinct pathways:

1. Cytoplasmic accumulation of NF-κB with p65 and p50 subunits as a result of protein synthesis, manifested by nuclear translocation.
2. p38 MAPK-dependent transactivation of NF-κB.

Both of these are known to be required for full activation of NF-κB-dependent transcription (24). Interestingly, the marked activation of angiogenesis in urinary bladder lamina propria observed in all cases of groups 1 and 2 with Chernobyl cystitis was associated with a dramatic increase of p38 MAPK and p65 cytoplasmic and nuclear expression as well as cytoplasmic iNOS and Cox2 in endothelial cells (Figure 3). These findings indicate a critical role for oxidative stress, as well as for the p38 MAPK signaling cascade and for the transcriptional factor NF-κB in endothelial cell activation, as an important component in the pathogenesis of Chernobyl cystitis. iNOS and Cox2 cytoplasmic expression in capillary endothelial cells of lamina propria, accompanied by the hypervascularization and the development of angiomatoid-like vessels, especially in group 1 patients, confirms the involvement of oxidative stress as a trigger of the angiogenic cascade, which is known to play a major role in human bladder carcinogenesis (25).

Furthermore, strong H-ras expression was evident in the bladder urothelium of patients from groups 1 and 2 (26). Numerous cytokines...
and growth factors can activate the ras gene and H-ras expression may affect cell responses to chemical inducers of oxidative stress (27). This means that the H-ras expression in our material might reflect not only H-ras oncogene activation in bladder urothelium as an early event of human bladder carcinogenesis but also angiogenesis activated by oxidative stress.

In contrast to our results, it has been recently suggested that oxidative stress might play a key role in the inhibition of tumor initiation by p38 MAPK. Thus, Dolado et al. (28) suggested that p38 MAPK acts to suppress tumor formation by H-ras. p38 MAPK pathway is implicated in suppression of tumorigenesis because it can inhibit cell growth by decreasing the expression of cyclin D (29), inhibit the activity of Cdc25 phosphatases (30) and engage the p16/Rb and p19ARF/p53 tumor-suppressor pathways (31–33).

In conclusion, our findings point to a significant relationship between oxidative stress induced by long-term, low-dose IR in people living ≥20 years in radio-contaminated (137Cs) areas of Ukraine and the development of Chernobyl cystitis, a possible pre-neoplastic condition in the urinary bladder.

**Fig. 3.** Immunohistochemical findings for a group 1 male with dysplasia (A–D) and small papillary urothelial carcinoma (E–G). (A) HE, (B) p65 expression, (C) p50 expression, (D) p38 expression, (E) HE, (F) p65 expression and (G) p50 expression. Magnification ×100.
**Growth factors**

Exposure of cells to a variety of stresses, including IR, induces compensatory activation of multiple intracellular signaling pathways. Change in expression of growth factor receptors such as fibroblast growth factor receptor 3 (FGFR3) and epidermal growth factor receptors (EGFRs) expressed by target cells due to radiation-induced signaling is therefore a question of interest. In our search for new markers of urinary bladder carcinogenesis, we recently were attracted by the FGFR3, a member of the receptor tyrosine kinase family, with heparin glycosaminoglycan as an important cofactor (34). Recently, FGFR3 mutations were found in >70% in non-invasive papillary urinary bladder carcinomas in humans (35). The high frequency of FGFR3 mutations in superficial urothelial carcinomas contrasts with the absence of FGFR3 mutations in CIS and the low percentage of mutations in invasive urothelial carcinomas (34,35).

Recent studies showed that EGFR1 expression is increased in muscle-invasive carcinomas as compared with superficial bladder carcinomas and correlates with poor survival of patients with bladder cancer (36). Moreover, the ErbB2 proto-oncogene, also called HER2 or EGFR2neu located at 17q21, which encodes a 185 kDa transmembrane growth factor receptor (p185HER2/neu) with tyrosine kinase activity, has been shown to be over-expressed in a variety of primary human carcinomas with a more aggressive clinical course, including breast, lung, ovarian and urinary bladder carcinomas (37).

Several signal transduction pathways have been implicated in EGFR-dependent cell survival as it relates to the anchorage-independent state. Primarily, these include the Ras/Raf/MEK/MAPK cascade. Raf-1 is a member of a family of serine–threonine protein kinases termed Raf-1, B-Raf and A-Raf, activated dependent upon Raf-1 translocation to the plasma membrane where phosphorylation occurs (38). Raf-1 activates MAP/ERK kinase (MEK), which in turn phosphorylates MAPK (39). Raf-1 protein expression is elevated in human breast carcinoma and it might be responsible for malignant progression (40). However, we were unable to find studies reporting the presence of Raf-1 protein in urinary bladder carcinomas, except one, related to Raf-1 amplification in urothelial cancers with mutation of the p53 gene (41).

On the basis of these observations, we designed a study to compare alterations in signaling cell systems in different patient groups (with pre-neoplastic lesions, Chernobyl cystitis and already well-developed urothelial carcinomas) and relationships among three immunohistochemical markers of the growth factor receptors and Raf-1.

Molecular analysis of some growth factor receptors in urinary bladder lesions, described by our group as Chernobyl cystitis (groups 1 and 2), as well as urothelium from analogous patients from so-called clean areas (group 3) with primary urothelial carcinomas, revealed dramatic nuclear and cytoplasmic FGFR3 (isotype unknown) over-expression with a high score of 95% in group 1 cases (42). Interestingly, multiple areas of dysplasia and CIS were stained consistently and very strongly. Furthermore, because the FGFR3 gene is known to be expressed both in normal urothelium and in bladder carcinomas, it is likely that the mutant gene has an oncogenic role in bladder carcinoma pathogenesis (35).

We have recently found FGFR3 gene mutations in 30% of cases in the urothelial carcinoma patients (A.Romanenko, K. Morimura, A.Kakehashi, H.Wanibuchi, A.Vozianov, and S.Fukushima, unpublished data), the majority within the spectrum of oncogenic mutations (codon 249 TCC to TGC). Interestingly, mutations were detected almost equally in both high- and low-stage urothelial carcinomas, in contrast to recent reports of an absence of FGFR3 mutations in CIS and a very low percentage of mutations in invasive (pT2-4) urinary bladder carcinomas (35).

The marked increase of FGFR3 nuclear expression in 95% of urothelia in group 1 patients, associated with dysplasias and CIS, could be strongly related to the early events of the urinary bladder carcinogenesis in people living in the radio-contaminated areas of Ukraine. The majority (70%) of the primary bladder carcinomas in the additional urothelial carcinoma group (also from patients living in the radio-contaminated areas) demonstrated nuclear, membranous and more rarely cytoplasmatic FGFR3 expression in both superficial and invasive carcinomas. These findings provide support for the idea that in the case of the presence (induction) of FGFR3 mutations, invasive carcinomas may arise from mutated superficial (pTa–pT1) carcinomas, which might progress from CIS.

It is necessary to note that FGFR3 cytoplasmic and nuclear over-expression was detected in both endothelial cells and fibroblasts of lamina propria of the same patients. Our results might suggest that mesenchymal cells are required for the induction of urothelial cell transformation. Evidence has accumulated that epithelial autocrine cells, constitutively producing and secreting the FGF and expressing FGFR3, have a highly aggressive mesenchymal phenotype (43). Other studies demonstrated that FGFs can activate FGFRs that in turn can promote tumor cell proliferation in an autocrine fashion by stimulating the growth, survival and migration of stromal cells, including fibroblasts and smooth muscle cells, or, as demonstrated in our work, participating in the induction of tumor angiogenesis (44). Importantly, the marked activation of angiogenesis in urinary bladder lamina propria in the majority of cases with Chernobyl cystitis was associated with a dramatic increase of FGFR3, EGFR1 and EGFR2neu expression in endothelial cells.

In our studies, EGFR1 and EGFR2neu immunoreactivity was found to be increased in the same cells of the bladder urothelium in group 1. Cytoplasmic over-expression in particular appears significantly associated with the Chernobyl cystitis pattern. This may also be the case for squamous cell carcinoma and pre-cancerous lesions of the lung (45), with prognostic importance for trafficking of EGFR1 between the Golgi apparatus and cell membranes and internalization of EGFR1 after interaction with an EGFR ligand. Moreover, cytoplasmatic expression of EGFR has been correlated with a poor prognosis and increased metastatic potential in lung cancers (45).

EGFR and other receptor tyrosine kinases are immediate early response gene products that are activated by IR within minutes, as with their physiological growth factor ligands that cause complex cytoprotective responses including increased cell proliferation, reduced apoptosis and enhanced DNA repair (46). As repeated radiation exposure can induce a strong cellular proliferative response in vitro (47), evaluation of human response levels after sustained long-term, low-dose IR is clearly of interest.

Cell–cell contact inhibition is considered to contribute to control of cell growth, with involvement of molecules that mediate cell adhesion, such as cadherins (39). In addition to our finding of significant EGFR1, EGFR2neu and Raf-1 activation in urothelium of group 1 patients, transforming growth factor (TGF)–α over-expression and alteration in E-cadherin–β-catenin complexes in the analogous bladder lesions has been reported (48). Since cell–cell contact inhibition is an important regulatory mechanism of cell growth (49), its loss due the MAPK activation could contribute to uncontrolled tumor growth and might correlate with an invasive phenotype (39). Collectively, our findings suggest that FGFR and EGFR signaling pathways associated with EGFR2neu and Raf-1 activation may contribute to several facets of multi-stage urothelial carcinogenesis, including auto- or paracrine growth stimulation, up-regulation of angiogenesis and stromal remodeling.

**DNA damage and repair**

Understanding the long-term effects of low doses of IR on living organisms requires identification of critical radiation-induced DNA lesions, measurement of their repair and determination of the consequences of mis-repair or non-repair. Our results suggest that increased oxidative stress in the bladder urothelium of the Ukrainian population in Chernobyl radio-contaminated areas is accompanied by marked DNA damage and repair by 8-oxoguanine-DNA glycosylase 1.

Our recent studies showed mutational inactivation of the p53 tumor-suppressor gene, with strong p53 immunoreactivity of urinary bladder urothelium in this group of Ukrainian patients (7). The p53 gene in vivo modulates the base excision repair activity pattern after the oxidative stress generated by IR (50), suggesting a direct role in
housekeeping base excision repair activity, with molecular cross-talk between the p53 protein and the DNA repair machinery. Furthermore, mutant p53 seems to confer higher base excision repair activity in response to oxidative stress (51), in agreement with our observations of strong base excision repair enzyme expression in areas of urothelial dysplasia and CIS. However, activity is irregular in the background urothelium so that some cells may suffer inefficient base excision repair (52). In contrast, nuclear over-expression of apurinic/apyrimidinic endonuclease 1 (APE1), a major endonuclease acting on apurinic/apyrimidinic sites after exposure to IR, appears homogeneous. Recently, nuclear APE1 expression was documented as a facet of increased cellular resistance to oxidizing agents (53), contributing to base excision repair of reactive oxygen species (ROS)-induced lesions. This finding is consistent with the previous observation that APE1 activation after ROS generation is accompanied by adaptive resistance of the cells to ROS (53). Our data suggest that APE1 is also important for repairing oxidative DNA lesions in human urothelium as a result of long-term, low-dose exposure to IR (52).

The significant increase of nuclear xeroderma pigmentosum A (XPA) protein level evident in urothelium of group 1 patients is indicative of activation of transcriptional coupled repair and global genome repair pathways. Recent studies showed a major role of XPA protein in the early steps of nucleotide excision repair reaction with low-dose (0.2–2 Gy) IR exposure (54). Interestingly, a marked increase in nuclear 8-OHdG, APE1 and XPA, but not 8-oxoguanine-DNA glycosylase 1, was detected in the endothelial cells of microvessels, macrophages and lymphocytes close to the urothelium, also suggesting DNA repair in the bladder propria mucosa. Importantly, all detectable small invasive transitional cell carcinomas in our group 1 patients were mostly negative for 8-oxoguanine-DNA glycosylase 1, APE1 and XPA presumably, indicating the apparent disruption of the base and nucleotide repair machinery in these malignant cells. Whether oxidative damage does not occur in tumors is a question that requires further investigation.

**Microenvironment—intercellular communications**

Redox homeostatic processes in the microenvironment, involving signaling pathways and interaction between cells and also with the extracellular matrix, could be targets of chronic low-dose IR. Cellular interactions are modulated by cytokines and growth factors, which are known to be multi-functional molecules that orchestrate most aspects of the inflammatory response, eliciting their effects locally or systemically in an autocrine or paracrine manner (55,56). One of the TGF family multi-functional cytokines, the TGF-betal (TGF-β1), is known to regulate cell growth and differentiation, tissue remodeling, immune response and angiogenesis. Three major isoforms exist in mammals: TGF-β1, 2 and 3. Alterations of cadherin–catenin complexes have also attracted interest as important for progression of many carcinomas in humans. Components of the E-cadherin–β-catenin complex, as well as TGF-β1 and iNOS, were evaluated in urinary bladder specimens in an attempt to detect molecular lesions of cellular membranes with a possible role in urothelial changes in patients chronically exposed to low-dose IR after the Chernobyl accident in Ukraine. We thereby documented for the first time that Chernobyl cystitis with foci of urothelial dysplasia, and CIS, and fibrosis of the lamina propria, is associated with activation of TGF-β1, as well as disruption of E-cadherin–β-catenin expression, which might be a result of chronic long-term, low-dose IR exposure.

Whereas excess ROS production clearly damages DNA, low levels might affect cell signaling, particularly, redox modulation. TGF-β1 is a major extracellular signaling sensor of damage, as it mediates redox homeostasis in cells and contributes to cell–cell communication (55). Interestingly, it is known as a mediator of tissue response to IR, pointing to a role in orchestrating changes due to oxidative stress (57). The present results indicate that alteration of TGF-β1 is a frequent event in background bladder urothelium of group 1 patients, with decrease observed in areas of dysplasia and CIS and little to no immunoreactivity in urothelial carcinomas. Furthermore, we found TGF-β1 immunostaining to be most pronounced in the lamina propria, in particular, in areas of sclerosis and fibrosis, where production of inflammatory cell cytokines is clearly important (56). IR appears to be one of a few exogenous agents that can cause TGF-β1 activation in situ.

Evidence has accumulated that gap junction intercellular communication is sensitive to oxidative stress (58). It was demonstrated that loss of Cx32/gap junction intercellular communication plays a significant role in radiation-induced tumorigenesis of the liver and importantly that Cx32 may also play a role in tumor suppression and/or tumor progression in other tissue types such as lung and adrenal gland (59). Cx32 was demonstrated to function as a hepatic tumor suppressor in response to radiation-associated mutation events (59). E-cadherin–β-catenin complexes are required for formation of gap junction intercellular communication, whereas mutations in the β-catenin gene have been described for various human cancers (60). Our findings indicate that alterations of E-cadherin–β-catenin are frequent in bladder urothelium of people living in radio-contaminated areas. Elevated protein levels of E-cadherin and β-catenin as well as their abnormal cytoplasmic localization detected in the urothelial cells could be the early event in urinary bladder carcinogenesis induced by long-term, low-dose IR exposure. Our results are in line with other studies have suggested that oxidative stress causes internalization of E-cadherin from the plasma membrane to the cytosol (48,61). Intracellular accumulation of β-catenin might correspond to an increased level of hypophosphorylated β-catenin caused by a mutation, in turn leading to transduction of oncogenic signals and cancer progression (62). Over-expression may result in the premature reentry of cells into the cell cycle after γ-irradiation-induced DNA damage and thereby promote the accumulation of oncogene mutations and carcinogenesis (63).

Importantly, aberrant expression of β-catenin and E-cadherin in association with TGF-β1 up-regulation in lamina propria appears to be essential molecular alteration in pathogenesis of bladder urothelial carcinomas in the environment of continued chronic long-term, low-dose IR exposure, when compared with CIS and urothelial carcinomas which developed before the Chernobyl accident.

**Ubiquitination and sumoylation**

Recent evidence suggests that the ubiquitin (Ub)/proteasome system may play a critical role in responses to IR, controlling numerous physiological processes including signal transduction, DNA repair, cell cycle progression, cell survival and stress responses (64,65). This is in addition to its more established roles in the removal of misfolded, damaged and effete proteins (64,65). Ub, consisting of 76 amino acids, is an element within the Ub/proteasome system requiring initial activation by Ub-activating enzyme E1 in an ATP-dependent reaction. Ub is covalently bound to E1 and then passed to the E2 Ub-conjugating enzyme, where it forms a similar thiolester linkage. Ubiquitinated proteins are then degraded by the 26S proteasome complex (64). In addition to Ub, there are several Ub-like proteins. One of them, the small Ub-related modifier (SUMO), has been shown to covalently modify a large number of proteins with important roles in many cellular processes including gene expression, maintenance of chromatin structure and signal transduction (66). Post-translational modification by SUMO is not associated with protein degradation but rather plays role in determining protein localization and activity. In contrast to the Ub system where dozens of E2 enzymes have been identified, Ub-9 is the only known SUMO E2-conjugating enzyme (67). Many known targets for SUMO1 and Ub9 (the integral players in sumoylation) are nuclear proteins with important roles in regulating transcription chromatin structure and DNA repair (68).

Classical examples are the tumor-suppressor proteins p53 and p27Kip1 whose function is abolished in many tumors. Since ubiquitination and sumoylation are intimately involved in major cellular biological processes and many effects of IR involve changes in protein stability, it is reasonable to ask what effect these processes have on cellular responses to chronic long-term, low-dose radiation.
Therefore, we examined the levels of ubiquitination and sumoylation elements in Chernobyl cystitis induced by long-term, low-dose IR in Ukraine. For this purpose, Ub, SUMO1 protein, the SUMO E2-conjugating enzyme Ubc9, the cell cycle inhibitor p27Kip1 and the tumor-suppressor protein p53 were evaluated by immunohistochemistry in a series of radiation-exposed patients and controls. This revealed Ub, SUMO1 and Ubc9 over-expression, with high scores in 96, 96 and 72% of cases, respectively (Table III). Steady-state accumulation of p53 protein in 60% cases was accompanied by loss or severe reduction of p27Kip1 protein in 76% cases (69). Over-expression of Ub, SUMO1 and Ubc9 might be related to the accumulation of the mutated p53, which is not degraded in IR-affected cells or, on the contrary, to the proteolysis of p27Kip1 tumor suppressor.

The Ub/proteasome system may have a critical role in rapid and late responses to IR with radiation-induced (through free-radical damage) impairment of proteasome function, which results in inhibition of proteolysis (70). Because repeat radiation exposure can induce a strong cellular proliferative response in vitro (71), human response levels after sustained long-term, low-dose IR are of interest.

The tumor-suppressor protein p53 is known to be rapidly turned over in unstressed cells by the Ub/proteasome-dependent pathway and it has been found to be covalently modified by SUMO1 in vitro and in vivo through direct interaction with the SUMO E2-conjugating enzyme Ubc9 (72). Accumulating evidence suggests an intrinsic role of sumoylation of p53 in stress-related processes, including DNA damage, oxidative DNA damage, and Ras oncogene activation.

Data are mean ± SD (immunohistochemical scores).

<table>
<thead>
<tr>
<th>Protein/factor</th>
<th>Groups 1 and 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>26</td>
<td>24</td>
</tr>
<tr>
<td>Contamination levels in soils (Ci/km²)</td>
<td>0.5–30</td>
<td>NC a</td>
</tr>
<tr>
<td>Ub</td>
<td>7.4 ± 1.9 b</td>
<td>1.7 ± 2.4</td>
</tr>
<tr>
<td>SUMO1</td>
<td>7.6 ± 1.8 b</td>
<td>4.4 ± 2.9</td>
</tr>
<tr>
<td>Ubc9</td>
<td>6.4 ± 2.4 b</td>
<td>2.6 ± 1.5</td>
</tr>
<tr>
<td>p53</td>
<td>5.1 ± 3.7 b</td>
<td>0.7 ± 1.0</td>
</tr>
<tr>
<td>p27</td>
<td>3.0 ± 2.0 b</td>
<td>6.5 ± 2.6</td>
</tr>
</tbody>
</table>

Table III. Immunohistochemical scores for proteins of Ub/proteasome system

Fig. 4. Schematic representation of cellular and molecular responses induced by exposure to chronic long-term, low-dose IR in the bladder urothelium of people living in 137Cs-contaminated areas of Ukraine after the Chernobyl accident.
damage (72). Therefore, the fact that at least 60% of group 1 cases showed high scores for p53 protein over-expression is of clear interest (69). Earlier, we reported defective regulation in cell cycling and DNA damage involving p53 and mdm2 expression, accompanied by specific p53 gene mutations with G-C to A-T transitions at CpG dinucleotides and a hot spot at codon 245 in 53% of a series of patients with Chernobyl cystitis (1,7). Therefore, our findings support the idea that increased p53 protein stability resulting from mutations prevents recognition by the Ub-mediated degradation machinery as well as by sumoylation modifying processes.

Destabilization of another Ub/proteasome target, the tumor-suppressor p27Kip1 protein, has also been implicated in the pathogenesis of cancers (64). Of our cases of Chernobyl cystitis, 36% showed loss or significantly decreased levels of p27Kip1 expression in the nuclei and cytoplasm. However, in 44% of cases, we observed predominantly p27Kip1 cytoplasmatic staining. These observations suggest that decreased nuclear p27Kip1 expression may be due to cytoplasmic translocation, known to be a result of mitogenic stimulation and has been claimed to be related to a poor prognosis (73).

Thus, our study suggests that ubiquitination and sumoylation processes are direct targets of long-term, low-dose IR exposure, leading to unscheduled protein degradation. Up-regulated ubiquitination and sumoylation processes might be an adaptive response to insufficient proteolysis of aberrant p53 and p27Kip1 occurring due to long-term, low-dose IR exposure. These results are in line with recent findings that aberrant, unscheduled proteolysis of many cell cycle regulators contributes significantly to tumorigenesis, with increased protein stability as a result of mutations that prevent recognition by the Ub-mediated degradation and sumoylation machinery (74). If critically damaged cells with aberrant p53 protein escape from elimination, this would mean survival of cells with mutations in an environment of continued long-term, low-dose IR exposure.

Conclusions

Our data support the hypothesis of distinct molecular carcinogenesis pathways for bladder cancer in Ukraine before and after the Chernobyl disaster (75). The biological effects of chronic low doses of IR and their relationships with chronic inflammation and carcinogenesis have received much attention in the last few years. Series of our recent studies point to a strong relationship between oxidative stress, accompanied by at least two signaling pathways (involving p38 MAPK, NF-κB cascades) and growth factor receptor activation, induced by the IR exposure of people who have lived for ~20 years in radio-contaminated areas of Ukraine, and the development of radiation chronic proliferative atypical cystitis, so-called Chernobyl cystitis, a pre-neoplastic condition in humans (Figure 4). Dramatic increase of iNOS, Cox2 and 8-OHdG expression is associated with the observed chronic inflammation (25); this may be mutagenic through NO-mediated DNA damage or hindrance to DNA repair and thus potentially carcinogenic, as suggested by p53 accumulation in urothelium of Chernobyl cystitis together with frequent G-C to A-T transitions at CpG dinucleotides in the p53 gene (7). Inflammation-induced reactive oxygen and nitrogen species cause damage to important cellular components (e.g. DNA, proteins and lipids), which can directly or indirectly contribute to neo-polymer plastic cell transformation. Over-expression, elevated secretion or abnormal activation of pro-inflammatory mediators, such as cytokines, chemokines, Cox2, prostaglandins, iNOS and NO, and a distinct network of intracellular signaling molecules including upstream kinases and transcription factors facilitate tumor promotion and progression. These enzymes may be acting as a ‘landscaping tumor promoters’ in the bladder urothelium and influence tumor growth according to the landscaping model proposed by Kinzler and Vogelstein (1998). On the other hand, DNA methylation and histone modifications are important epigenetic mechanisms of gene regulation and play essential roles both independently and cooperatively in tumor initiation, promotion and progression. Furthermore, recently, the possible mechanisms by which inflammation can contribute to carcinogenesis were reported to include induction of genomic instability, alterations in DNA methylation and subsequent inappropriate gene expression, enhanced proliferation of initiated cells, resistance to apoptosis, aggressive tumor neo-vascularization, invasion through tumor-associated basement membrane metastasis etc. (76). Furthermore, significant alteration in regulation of cell cycle transition associated with increased activation of proliferative processes (p53, cyclin D1, mdm2, p21WAF1/Cip1 and PCNA protein over-expression) has been documented (9), along with changes in ubiquitination and sumoylation processes in association with aberrant unscheduled proteolysis of many cell cycle regulators (69). Moreover, DNA repair appears to be an early event in the radiation-induced adaptive response. Exhaustion of the capacity for base and nucleotide excision repair pathways associated with Chernobyl cystitis may be related to the carcinogenic potential of the urothelial lesions.

Bladder urothelium as well as endothelial cells, fibroblasts and immune cells in the microenvironment respond to chronic low-dose IR by activation of FGFR and EGFR signaling pathways in association with EGFR2 neu, Raf-1 and stromal TGF-β1 activation with aberrant expression of the urothelial β-catenin–E-cadherin complexes, which may potentially contribute to several facets of multi-stage urothelial carcinogenesis, including auto- or paracrine growth stimulation, up-regulation of angiogenesis and stromal remodeling. It can be supposed that the microenvironment changes induced by IR, detected in our studies, could promote angiogenesis, remodeling the extracellular matrix to facilitate invasion as well as the progression of pre-existing initiated cells to malignancy. This hypothesis for chronic long-term, low-dose IR action is supported by the emerging concept that elements other than epithelial cells, and mechanisms other than genetic alteration, influence the processes of carcinogenesis (77). Based on now available IR-induced urinary bladder carcinogenesis markers in patients living in radio-contaminated areas, prediction and early detection of Chernobyl cystitis becomes very important. Moreover, patients from the radio-contaminated areas have to be followed up for many years with the cytological analysis of urine sediment as well as other clinical investigations. The targeting therapy might be used against some genes, growth factors and other important molecules, which are responsible for cell cycle transition, cell–cell communication and signaling cascades. Furthermore, it might be recommended to use some pectines and able to bind to 137Cs and other radionuclides, thus eliminating them. Placing chronic low-dose radiation damage at the cellular level into the context of a dynamic multicellular system will provide a better basis for understanding and treatment of Chernobyl cystitis—the first step in a specific category of urinary bladder carcinogenesis in humans.

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