Urinary bladder lesions induced by persistent chronic low-dose ionizing radiation

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The incidence of urinary bladder cancer in the Ukraine increased from 26.2 to 43.3 per 100 000 population between 1986 and 2001 after the Chernobyl accident. The present study was conducted to evaluate the development of radiation-dependent lesions in the urinary bladders of people living in cesium 137 (^{137}Cs) radio-contaminated areas of the Ukraine. Bladder urothelial biopsies from 159 male and 5 female patients were subjected to histological examination and immunohistochemical study of p38 mitogen-activated protein kinase (MAPK), as well as the p50 and p65 subunits of nuclear factor kappa B (NF-κB). A pattern of chronic proliferative atypical cystitis accompanied with large areas of sclerosis of connective tissue in the lamina propria was commonly observed in all cases. Interestingly, these lesions were associated with a dramatic increase in the incidences of dysplasia/carcinoma *in situ*, and, moreover, small urothelial carcinomas were incidentally detected. We defined the overall condition as "Chernobyl cystitis." Greatly elevated levels of p38, p65 and p50 expression in the urothelium were evident and the patients showed increased ^{137}Cs in urine. The data support conclusions from our previous studies of a critical role for increased oxidative stress in generation of urinary bladder urothelial lesions in individuals chronically exposed to low-dose ^{137}Cs radiation. Alterations in the p38 MAPK cascade and accumulation of NF-κB subunits could be crucial early molecular events in the pathogenesis of Chernobyl cystitis. (Cancer Sci 2003; 94: 328–333)

The Chernobyl accident which occurred in April 1986 in Ukraine, is unique not only as the largest catastrophe in the history of nuclear power utilization, but also as the first to pose the problem of chronic long-term effects of low-dose ionizing radiation (IR) in humans. Towards the end of the second post-Chernobyl decade a dramatic increase in the incidence of urinary bladder cancers in Ukraine, from 26.2 to 43.3 per 100 000 population between 1986 and 2001, is now attracting strong public attention.[1)] More than 17 million people who live in the radio-contaminated areas of Ukraine, Russia and Byelorussia are being continuously exposed to low doses of still existing cesium 137 (^{137}Cs) radiation, known to account for 90% of internal radioactivity, which is concentrated and eliminated through urinary excretion.[2–5)]

Our previous study revealed that the majority of men with benign prostatic hyperplasia (BPH) living in radio-contaminated areas of Ukraine also exhibited multiple urothelial dysplasias, carcinoma *in situ* (CIS) and even small urothelial carcinoma (UC).[6)] Also we earlier demonstrated some manifestations of defective regulation in cell cycling and DNA damage involving specific *p53* mutations with G:C to A:T transitions at CpG dinucleotides, and a hot spot at codon 245 in 53% of examined patients, associated with markedly elevated levels of inducible nitric oxide synthase (iNOS), cyclooxygenase 2 (COX2) and 8-hydroxy-2′-deoxyguanosine (8-OHdG). These findings indicate increased oxidative stress in the urothelium, apparently accompanied by strong over-expression of p53 and H-ras.[6–8)] Until now, however, we have not unequivocally confirmed that the ^{137}Cs radionuclide itself really plays crucial role in the development of the post-Chernobyl urothelial lesions.

The term "low dose" means above background levels (0.1–0.5 Gy of radiation), yet below that which could induce acute effects usually associated with cell death.[9)] Low-dose IR has been recognized to act as a mitogen as well as a generator of free radicals through oxidative processes, which are not significantly different from those occurring during normal oxidation.[10)] Recent studies have shown that low doses of radiation can not only initiate carcinogenesis, but also act as a promoter or progressor, especially when the exposure is regular and sustained.[9, 11, 12)] Accumulating evidence indicates that free radicals such as reactive oxygen species (ROS) and nitric oxide (NO) or its derivatives are key determinants in carcinogenesis.[13, 14)] However, the crucial mechanisms leading to activation of transcription processes and mRNA expression that promote development of focal lesions in urinary bladder urothelium of people living in radio-contaminated areas remain to be elucidated.

There is evidence that ROS can act as activators or transcription factors, either directly or indirectly by activating other signaling cascades.[15)] The p38 mitogen-activated protein kinase (MAPK) pathway is involved in primary signaling stimulated by inducers of stress. The prototypic member of this group, p38a (also known as p38, CSBP or Rk), contributes to early stress responses activated by agents such as heat, UV, IR and inflammatory cytokines, resulting in inhibition of cell proliferation and eventual cell death.[16, 17)] Recent studies have demonstrated that activation of the transcriptional factor, nuclear factor kappa B (NF-κB), also plays an essential role in oxidative stress cascades. NF-κB consists of two major subunit-polypeptides, p50 and p65, and exists in cells as an inactive cytoplasmic precursor in complexes with the inhibitor-kappa B. Stimulation triggers its release, resulting in translocation from the cytoplasm to the nucleus, where NF-κB binds to DNA and regulates specific gene transcription.[18–21)] 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.[20–22)] It has been demonstrated that ^{137}Cs γ-rays at low dose (1.17 Gy/min) cause expression of NF-κB in human B-lymphocyte precursor cells.[23)] However, the precise mechanisms and the significance in this context of long-term (about 16 years), low-dose exposure to ^{137}Cs in the human urinary bladder urothelium are not yet known.

In the present study, histological patterns and the pathogenesis of chronic cystitis associated with long-term low dose radiation exposure in the Ukrainian population were investigated. In addition, p38 and NF-κB proteins were also analyzed as oxidative stress markers in an attempt to cast light on mechanistic aspects.

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Materials and Methods

Patients and urinary bladder samples. Formalin-fixed, paraffin-embedded tissue blocks from 159 male patients without any symptoms of urinary bladder disease, who underwent open transbladder prostatectomy for BPH and from 5 female patients with symptoms of chronic cystitis that were treated at the Institute of Urology in Kiev, Ukraine, were investigated. All patients gave their written informed consent and the study was approved by the Institute of Urology Ethics Committee (Kiev, Ukraine). Patients' characteristics are summarized in Table 1. Group I individuals were from heavily contaminated areas, group II from the less contaminated city of Kiev and group III, the controls, from "clean" areas (without indicated radio-contamination but with possible chemical contamination, as all of the Ukraine is considered to be an ecological disaster area). These patients resided in the same areas during the pre- and post-Chernobyl accident periods. Radiometric measurement of ^{137}Cs was conducted with urine collected over 24 h from all patients of groups I–III before surgery was performed using a Standard Y-Radiometer RUB-01 (No. 2980, Moscow, Russia). The scintillation block NaI was used as detector with power resolution on a γ-ray spectrum for ^{137}Cs 662 keV of 7.8% at 100–2200 keV. Calibration of the spectrometer was carried out by the Ukrainian Center of Standardization and Metrology. Tests were conducted in Marinelli vessels in a volume of 1–1.2 liters. Before measurement, a test of the background was carried out with 1 liter of distilled water. In order to determine the average square error each measurement was carried out in triplicate over 8 h. Multiple mapping biopsies of the bladder urothelium (including areas of bladder neck and both ureter orifices) were taken from every patient. A total of 492 paraffin-embedded specimens were histologically investigated. The size of bladder epithelium samples was between 0.1 and 0.4 cm.

Histopathology and immunohistochemical staining. Sections, 4 to 5 μm thick, of bladder samples were stained with hematoxylin and eosin (HE). Bladder lesions were classified in accordance with the histological typing defined by the new classification of the World Health Organization International Classification of Urinary Bladder Tumours.[24] Immunohistochemical (IHC) staining of 41 cases collected during 1999–2001 (group I, 13; group II, 19; and group III, 9 cases) from a total of 164 cases was performed using the standard avidin-biotin peroxidase complex (ABC) method with a Vectastain ABC Elite kit (Vector, Burlingame, CA). Serial sections were deparaffinized, and after endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water for 5 min, they were microwaved in citrate buffer (pH 6.1) for 30 min, for antigen retrieval. Non-specific binding was blocked with 5% normal horse serum in phosphate-buffered saline (PBS) at room temperature for 30 min. Incubation was performed with anti-mouse human p38 (D-8), and anti-mouse human NF-κB p65 (F-6) monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:800 dilutions and anti-mouse human NF-κB p50 (E-10) monoclonal antibody (Santa Cruz Biotechnology) at 1:200 dilution overnight at 4°C. Sections of tissue known to be positive were used as controls; for negative controls, exposure to the primary antibody was omitted. Color was developed using 3,3′-diaminobenzidine, and microscopic evaluation was performed in a blind fashion without knowledge of the patient group. Consecutive serial sections were used for IHC and HE staining. All specimens were examined independently by at least 2 pathologists. Approximately 12–15 sections per case were analyzed.

Quantitative analysis. Quantitative estimation of IHC was performed according to a system for evaluation and grading of immunostaining patterns with multiple values for extent and intensity, giving scores of 0 to 9.[12] The extent of staining was scored on a semi-quantitative scale of 0 to 3, using the following criteria: 0, no detectable staining; 1, <10% scattered cells; 2, >10% but <50% stained cells; 3, homogeneous staining in >50% of cells. The intensity of staining was similarly scored: 0, no detectable staining; 1, weakly stained cytoplasm (or nuclei); 2, moderately stained cytoplasm (or nuclei); 3, strongly stained cytoplasm (or nuclei). Final scores were derived from multiplication of extent by intensity.[25]

Statistical analysis. The significance of differences between groups for mean values of ^{137}Cs levels in urine was analyzed using the Steel-type separate ranking test (SAS system; Release 6.12, SAS Institute, Inc., Cary, NC). The Fisher's exact probability and χ^2 tests were carried out to assess the significance of differences between groups for incidence (Stat View SE+

Table 1. Patients' characteristics

	Group I	Group II	Group III
No. of patients (females)	73 (1)	58 (4)	33 (0)
Median age (range)	65 (52–91)	72 (30–87)	66 (54–75)
Nutrition	standard	standard	standard
Cigarette smoker (%)[3]	22 (30.1)	34 (58.6)	11 (33.3)
Years of surgery	1996–2001	1996–2001	1996–2001
Contamination levels in soils (Ci/km^2)[1]	5–30	0.5–5	NC[2]

1) Data from Raes *et al*. (1991).
2) Non-contaminated.
3) For more than 10 years.

Table 2. ^{137}Cs levels in urine

	Group I	Group II	Group III
No. of patients examined	55	53	12
Contamination levels in soils (Ci/km^2)[1]	5–30	0.5–5	NC[2]
^{137}Cs levels in urine (Bq/liter)	6.47±14.30[3, 4]	1.23±1.01[4]	0.29±0.03

1) Data from Raes *et al*. (1991).
2) Non-contaminated.
3) Mean±SD.
4) Significantly different vs. group III at $P<0.001$ (Steel type separate ranking test).

Table 3. Incidence of urinary bladder dysplasias and carcinomas

Groups	No. of cases	Dysplasia (%)	Carcinomas		
			Total (%)	CIS[1]	Papillary UC[2]
I	73	71 (97)[3]	53 (73)[3]	47[3]	6
II	58	48 (83)[3]	37 (64)[3]	34[3]	3
III	33	9 (27)[4]	0 (0)	0	0

1) Carcinoma *in situ*.
2) Urothelial carcinoma.
3) Significantly different vs. group III at $P<0.0001$ (χ^2 or Fisher's exact probability test).
4) Mild dysplasia.

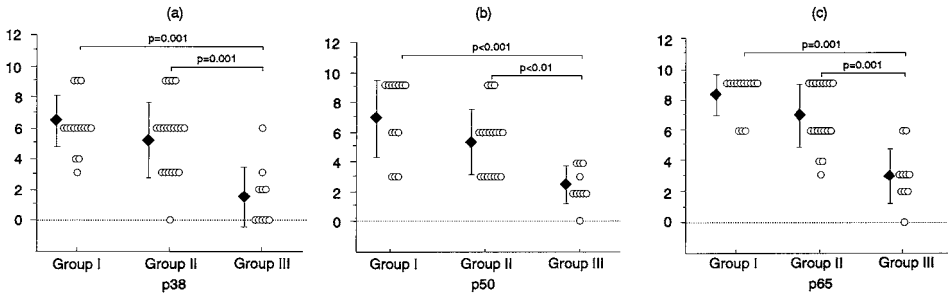

Fig. 1. IHC scores for urinary bladder urothelium in groups I through III for (a) p38, (b) p50, (c) p65. Vertical bars, mean±SD. The Bonferroni/Dunn test was applied for statistical analysis.

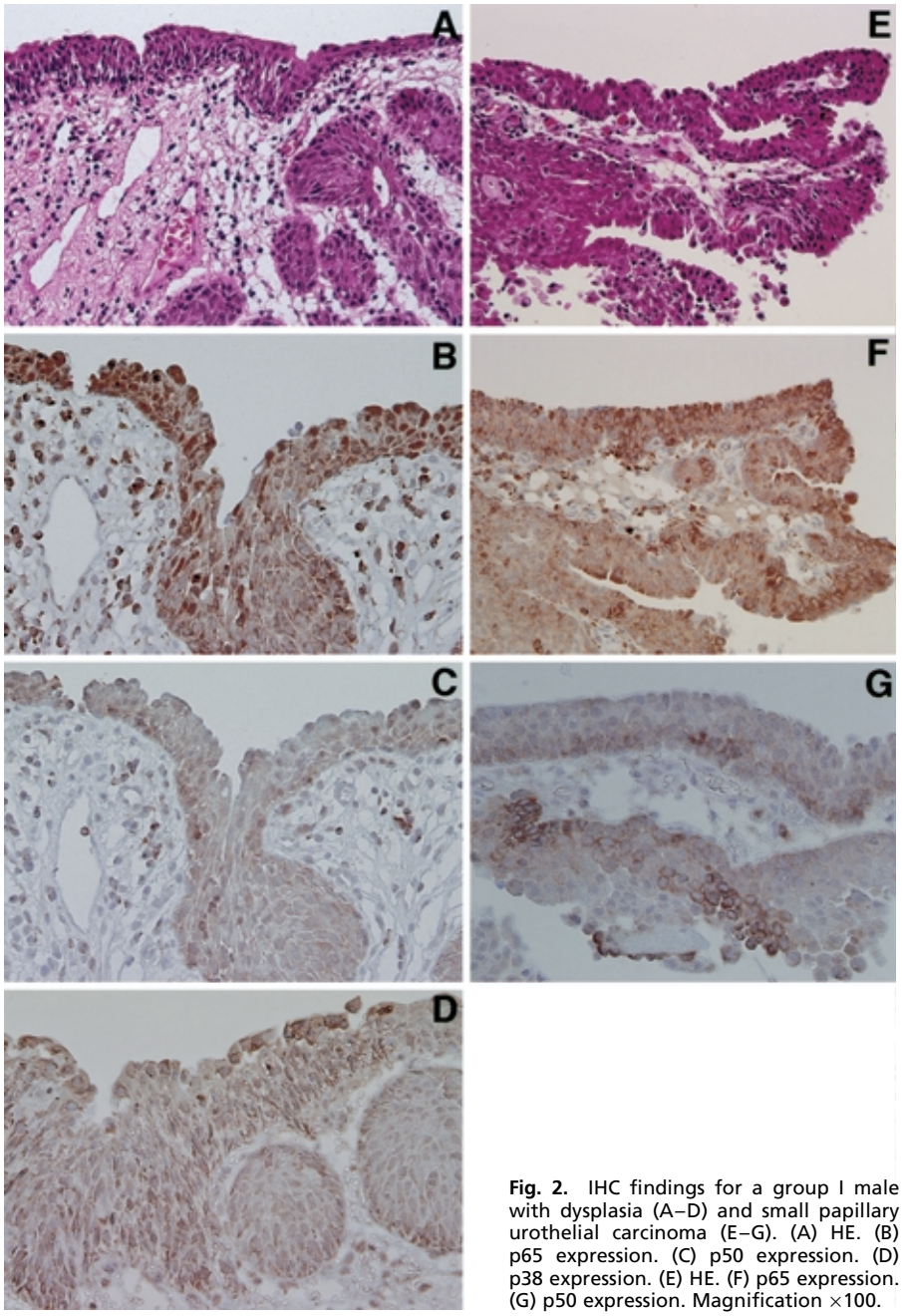

Fig. 2. IHC findings for a group I male with dysplasia (A–D) and small papillary urothelial carcinoma (E–G). (A) HE. (B) p65 expression. (C) p50 expression. (D) p38 expression. (E) HE. (F) p65 expression. (G) p50 expression. Magnification ×100.

Graphics, Abacus Concepts, Inc., Berkeley, CA) and the Bonferroni/Dunn test for immunohistochemistry data (Super ANOVA, Abacus Concepts, Inc.).

Results

Patients. The results obtained from ^{137}Cs measurement in 1-day urine from patients in groups I–III are summarized in Table 2. Significant elevation of ^{137}Cs levels was evident in those from group I (6.47 Bq/liter or 102 micro Sievert/year) and to a lesser extent from group II (1.23 Bq/liter or 19 micro Sievert/year), as compared with group III (0.29 Bq/liter or 5 micro Sievert/year).

Histopathology. The incidences of urinary bladder dysplasias and carcinomas in patients of the different groups are summarized in Table 3. Urinary bladder epithelium biopsied from 126 male patients with BPH from groups I and II demonstrated multiple areas of dysplasia with strong epithelial abnormalities such as extensive cellular pleomorphism and hyperchromasia, frequently combined with thickening of epithelium. The cells were large with abundant cytoplasm and prominent nucleoli in large nuclei. Foci of dysplasia were observed in 97% and 83% of cases in groups I and II, respectively. 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% of patients in groups I and II. Nine small papillary or invasive urothelial carcinomas were also incidentally found in individuals from radio-contaminated areas. Foci of mild dysplasia (4 of 5 cases) and CIS (2 of 5 cases) were detected in female patients of groups I and II.

All cases in groups I and II exhibited proliferative cystitis, i.e., von Brunn's nests, cystitis cystica, and squamous and glandular metaplasia, that were frequently combined and had marked features of chronic radiation cystitis rather than simple inflammation. Large areas of sclerosis and hyalinosis of connective tissue in lamina propria with less prominent inflammatory cellular infiltration of lymphocytes, macrophages, histiocytes and plasma cells were typical. Along with 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% in groups I, II and III, respectively. Endothelial proliferation of new microvessels in lamina propria was markedly increased in 46% and 34% of cases in groups I and II. In all female patients, large areas of sclerosis of connective tissue with some inflammatory infiltration and large areas of hypervascularization and patchy hemorrhage were detected in the lamina propria.

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 III. However, hypervascularisation and development of angiomatoid-like vessels were not observed.

Immunohistopathology. IHC results for p38 and NF-κB (subunits p50 and p65) of the urinary bladder urothelium in groups I through III are shown in Fig. 1. Representative patterns of histological and IHC staining in areas of dysplasia, CIS and small developing papillary urothelial tumor are illustrated in Figs. 2 and 3.

p38 expression: Average scores were 6.7, 5.0 and 1.8 in groups I, II and III, respectively. There was no significant difference (P=0.1187) between groups I and II, but both showed significant elevation (P=0.001) compared with group III (Fig. 1a).

The majority of specimens from groups I and II showed moderate and strong regular homogenous or granular immunostaining of nuclei and cytoplasm. Strong immunostaining was detected in 3 (23%) cases of group I and 4 (21%) cases of group II. Moderate cytoplasmic immunostaining was predominant in 9 (69%) and 9 (47%) cases of groups I and II, respectively, and slight immunostaining in 1 (7.6%) and 6 (32%) cases. Immunostaining was most pronounced in basal cells, but was partly positive in superficial layers of urothelium in patients of groups I and II (Fig. 2D). The majority of cases of group III were scored as 2 (40%) or 0 (40%). The other two cases (10% each), were scored as 6 and 3.

Strong nuclear overexpression of p38 was observed in lymphocytes, macrophages, histiocytes and especially endothelial cells of the microvessels and angiomatoid-like vessels in the lamina propria mucosa close to urothelium (Fig. 3F).

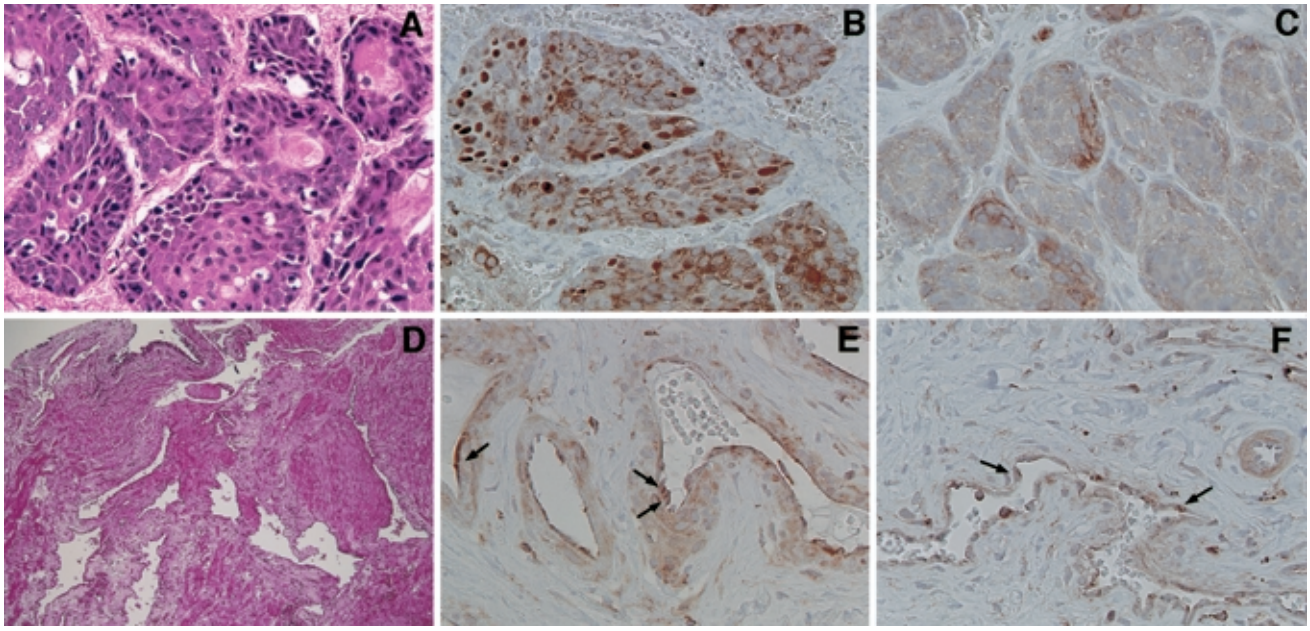

Fig. 3. IHC findings for a group II male with CIS, squamous metaplasia and invasive growth (C) in von Brunn's nests (A–C) and angiomatoid-like vessels (D–F) in sclerotic lamina propria. (A) HE. (B) p65 expression. (C) p50 expression. (D) HE. (E) p65 expression. (F) p38 expression. Magnification ×100.

p50 subunit expression: Average scores were 7.0, 5.2 and 2.5 for groups I, II and III, respectively. Significant differences were obtained for group I vs. group III ($P<0.001$) and for group II vs. group III ($P<0.01$) (Fig. 1b). In group I, 7 (54%) cases were scored as 9, 3 (23%) as 6 and 3 (23%) as 3. The strongest granular or homogenous cytoplasmic staining was detected in the basal layer with lower intensity in intermediate layers of the urothelium (Figs. 2C, G and 3C). Areas of dysplasia and CIS were p50-positive and were scored as 6 and 9. Nuclear staining in the basal layer was found in 2 cases only. However, the majority of cases from group II showed scores of 6 (42.1%) or 3 (36.8%), and only 3 (15.8%) were scored as 9. Areas of squamous metaplasia were mostly p50-negative.

The scores for p50 staining in group III were very low, 3 (30%) being scored as 4, 1 (10%) as 3 and 5 (50%) cases as 2.

p65 subunit expression: Average scores were 8.4, 6.9 and 3.0 in groups I, II and III, respectively. The difference between groups I and II was not significant ($P=0.0527$), in contrast to the cases for group I vs. group III ($P=0.001$) and for group II vs. group III ($P=0.001$) (Fig. 1C).

Strong granular or homogenous cytoplasmic immunostaining was observed in 10 (77%) of 13 and 8 (42%) of 19 in groups I and II, respectively. In 2 cases of group II, strong nuclear staining in basal and intermediate layers of urothelium was detected (Figs. 2B, F and 3B). The intensity was greatest in areas of dysplasia and CIS, which were scored as 9. Moderate staining (score of 6) was detected in 3 (23%) and 7 (37%) cases of groups I and II, respectively.

In p65-positive specimens of groups I and II, strong cytoplasmic and nuclear staining was evident in endothelial cells of microvessels, dilated and angiomatoid-like blood vessels, and macrophages, lymphocytes, histiocytes in lamina propria close to urothelium (Fig. 3E).

Discussion

In this report, we have documented for the first time that chronic, long-term (maximum about 16 years after the Chernobyl accident), low-dose ionizing radiation leads to the development of a previously unknown urinary bladder disease, radiation-induced chronic proliferative atypical cystitis or so-called Chernobyl cystitis, in humans. This is characterized by multiple areas of dysplasia and CIS of the urinary bladder urothelium in strong association with sclerosis and hyalinosis of connective tissue and strongly increased angiogenesis without a marked inflammatory reaction.

Our present radiometric study showed significant increase of ^{137}Cs in urine of patients from groups I and II, which suffered from BPH and presumably therefore urinary retention, so that radiation exposure of the urothelium would be expected to have been enhanced. ^{137}Cs γ-radiation levels in urine of group 1 patients (6.5 Bq/liter or 102 micro Sievert/year) were 20 times higher than in control group 3. 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 I and II, which were without urinary retention but with increased ^{137}Cs in urine, also demonstrated the same pattern of chronic proliferative atypical cystitis with less frequent mild dysplasia. These data strongly suggest the same pathway for development of urinary bladder lesions in both male and female patients, dependent on long-term exposure to low-dose ionizing radiation. However, it is necessary to add that Ukraine is acknowledged to be an ecological disaster area. The economic and social structures are very poor, and the majority of the Ukrainian population still get food from their private vegetable gardens (without any ecological control). Therefore, our patients (many of whom are smokers; Table 1) living in the radio-contaminated areas could be synergistically effected by various hazards. The critical role of the Chernobyl accident (involving long-lived ^{137}Cs and possibly many other radionuclides, which were not calculated in our study) as a part of this synergistic effect for the development of Chernobyl cystitis was evident, because these specific pathological lesions were not detected in analogous patients from so-called "clean" areas of the Ukraine, or in analogous patients from Sweden and Austria.[26]

Moreover, the biological effect of chronic low doses of IR and its relationship with chronic inflammation and carcinogenesis have received much attention in the last few years.[27] Our recent study showed a dramatic increase of iNOS expression associated with chronic inflammation in the bladder urothelium of people living in radio-contaminated areas of Ukraine[6]; this may be mutagenic through NO-mediated DNA damage or hindrance to DNA repair, and thus potentially carcinogenic.[28]

It is important to note also that there is no dose-response relationship in the range of 0.1 to 1 Gy, suggesting that an unstable phenotype may be induced by a quite low radiation dose.[29]

ROS produced by IR, have been implicated in the pathogenesis of cancer,[9] in the long term causing specific molecular changes that result in the activation or inactivation of transcription factors, which may alter gene expression. Key effects of IR include genotoxicity, lipid peroxidation, activation of activator protein 1 or NF-κB and generation of *p53* or *ras* gene family alterations.[15, 30] Our recent studies showed frequent and specific mutations of the *p53* gene, preferentially G:C to A:T transition at CpG dinucleotides, with a codon 245 hot spot in bladder urothelium under conditions of IR-associated oxidative stress.[6, 8] Moreover, this could lead to selective activation of the p38 MAPK cascade and mitotic arrest without apoptosis.[31] In this context, it is of interest that recent studies showed a strong correlation between COX2 mRNA overexpression, increased activity of the p38 stress-activated protein kinase and activation of NF-κB in MDA-MB-231 mammary carcinoma cells and U937 human macrophages.[32, 33]

The marked overexpression of p38 MAPK, p65 and p50 subunits of NF-κB in the same cells of the urothelium in the present study strongly indicates a pivotal role of oxidative stress in bladders of patients suffering long-term low dose radiation exposure. Moreover, ^{137}Cs radiation is known to cause a dose-dependent increase in mutants in an MN murine tumor cell line and to cause DNA strand breaks through generation of hydroxyl radicals.[34, 35] Together with our previous finding of strong cytoplasmic COX2 and iNOS overexpression in the urothelium,[6] the present results point to low-dose radiation exposure eliciting at least two distinct pathways:

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

Both of these events are known to be required for full activation of NF-κB-dependent transcription[36] and the concept is supported by recent evidence that ^{137}Cs γ-rays at the low dose rate of 1.17 Gy/min modulate NF-κB DNA binding activity in human lymphoblastoid cells with differential regulation of NF-κB subunit protein levels.[23]

Interestingly, the marked activation of angiogenesis in urinary bladder lamina propria in all cases of groups I and II with chronic radiation cystitis was associated with a dramatic increase of p38 and p65 cytoplasmic and nuclear expression in endothelial cells. These findings, in line with our previous study,[6] indicate a critical role for the p38 MAPK cascade and the NF-κB p65 subunit in endothelial cell activation, as an important component in the pathogenesis of Chernobyl cystitis.

It should be noted that classic descriptions of acute and chronic radiation effects on the urinary bladder do not include the pathogenesis of human urinary bladder injury after long-term (16 years), low-dose exposure to ionizing radiation. In our

patients, we did not observe reactive epithelial proliferation associated with fibrin deposits, fibrinoid vascular changes and multinucleated stromal cells—typical changes secondary to high doses of ionizing radiation.[24)]

In conclusion, our data point to a strong relationship between long-term, low-dose ^{137}Cs radiation exposure of people, who have lived for about 16 years in radio-contaminated areas of Ukraine, and the development of Chernobyl cystitis, a possible preneoplastic condition in humans. Our study suggests that the activation of two pathways, involving p38 MAPK overexpression and dramatic accumulation of NF-κB subunits induced by ROS in bladder urothelium could be crucial early molecular events, which may offer unique avenues for preventive or therapeutic intervention, as well as providing new insights into the mechanisms of urinary bladder carcinogenesis in humans.

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