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POMC expression of the urothelium of the urinary bladder of mice submitted to pelvic radiation

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Abstract

Objective: Patients who have had pelvic radiotherapy as part of their cancer therapy may develop subsequent urinary bladder injury. The acute changes that the urothelium undergo after radiation are known, but the healing mechanism of the urothelium of the urinary bladder after pelvic radiotherapy is not clearly understood. Proopiomelanocortin (POMC) peptides, which have immunomodulatory effects, are produced locally in sites outside of the central nervous system. This study aims to determine the role of POMC expression in the urothelium during radiation injury.

Methods: Twenty-four male Swiss Albino mice were divided into four groups. A single-fractioned 10 Gy of ionizing radiation was applied to the pelvic zone of all mice with Cobalt-60 radiotherapy. The first group 1, which consisted intact animal and not irradiated was the control group, and the second, third, and fourth groups were euthanized after 24 h (Group 2), 48 h (Group 3), and 7 days (Group 4) after irradiation. All bladders were prepared for histochemical analysis using hematoxylin eosin (H&E) and immunohistochemical analysis using anti-POMC antibody.

Results: No morphological differences were seen in all the group samples stained with H&E. POMC expression of the urothelium of bladder tissue samples shows different staining levels. Group 1 (96.7 ± 7.68), Group 2 (88.3 ± 8.04), and Group 3 (85.10 ± 10.9) were very weakly stained, but the POMC immunoreactivity of Group 4 (113.0 ± 12.8) was observed to be strong.

Conclusion: Expression of POMC from urothelium seems to prevent bladder damage from radiation supplying differentiation and restoration of the urothelium.

Keywords

bladder, histochemistry, immunohistochemistry, proopiomelanocortin, urothelium

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Introduction

Radiotherapy is often used in the treatment of different pelvic tumors, including those of the prostate, rectum, cervix, and endometrium. Adverse radiation-induced effects, however, may occur in nearby normal tissues, such as the bladder.[1] The effects of incidental irradiation on normal structures can occur within days to weeks, to months to years after exposure. Barcellos et al. reported that radiation-induced modifications in the bladder wall are time-dependent.[2]

The urinary bladder collects urine and functions as a temporary reservoir for urine. The bladder is covered by urothelium that consists of three to seven layers of transitional cells. It is known that

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radiation leads to inflammatory changes in the bladder, in which almost all cells are involved.[3] Two phases of radiation-induced changes in the urinary bladder are observed, an early phase occurs 2–6 weeks after the start of fractionated irradiation, which is characterized morphologically by hyperemia and mucosal edema. A chronic phase developing with latent times are dose-dependent, and can last for 10 years or longer. The morphological changes correlating in the initial late phase is a progressive mucosal breakdown, ranging from superficial denudation to ulceration, and even the formation of fistulae. The urothelial changes are accompanied by urothelial areas of compensatory hyperproliferation.[4] The radiation effects in the bladder start during treatment and resolve within a few weeks after the end of radiotherapy.

Proopiomelanocortin (POMC) is the polypeptide precursor of ACTH. There is a single POMC gene per haploid genome in humans. It is located on chromosome 2p23. It comprises 7665 bp and consists of three exons and two introns. In the normal human pituitary gland, the POMC gene is only expressed in corticotroph cells.[5] Outside the neuroendocrine tissue, POMC is present in the placenta, and in the immune system cells. In addition this, POMC peptides were observed in the skin epithelial cells, such as epidermis and also dermal infiltrating cells. POMC expression and POMC mRNAs were stronger in inflamed epidermis than in normal skin, and suggested POMC peptides have "a local stress reponse system" function in the skin.[6]

In this study, we aimed to elucidate the POMC expression of the urothelium of bladder before and after radiation exposure, using immunohistochemistry. The results obtained show the existence of a POMC expression, and confirm the hypothesis that the urothelium of bladder has a local protective system against radiation mediated by the POMC peptides.

Materials and methods

In this work, a total of 24 healthy, male, adult Swiss Albino mice, weighing 30–40 g, were obtained from Gazi University's Experimental Animal Laboratory and used as subjects. The subjects were isolated from stress and noise and fed with water and food *ad libitum* at 25°C in a cycle of 12 h of dark and 12 h of light before being included in the study. The animals were divided into four groups; the first group 1, which consisted intact animal and not irradiated was the control group, and the second, third, and fourth groups were euthanized after 24 h (Group 2), 48 h (Group 3), and 7 days (Group 4) after irradiation. Except for the control group, all mice were exposed to ionizing radiation in their pelvic region on the same day with the Co-60, 780-C device present in the Department of Radiation Oncology of Gazi University School of Medicine, applied to pelvic area with a source-to-surface distance of 80 cm for 10.7 min with a 10Gy dose for D_{max} in a single fraction. Ketamine was used a dose of 45–50 mg/kg intramuscular for sedation prior to radiation procedure. Euthanasia was performed on all the animal by cardiac puncture blood collection. After the euthanasia, the pelvic regions were dissected and the bladders were completely removed.

All bladder samples were first washed in a solution containing 10% formol and then placed in screw-cap sampling containers containing 10% formol. The tissue samples taken after the procedure were embedded into paraffin blocks. Serial sections (5 μm thick) were cut and prepared for both histochemical and immunohistochemical staining. The first sections were stained with hematoxylin and eosin (H&E) for histochemical examination. The second sections were used for immunohistochemical staining.

Immunohistochemistry

Formaline-fixed, paraffin-embedded bladder sections were used for immunohistochemical staining. Tissue samples were stored at 60°C overnight and then dewaxed with xylene for 30 min. After dehydration of the sections with ethanol, they were washed with distilled water. Subsequently, the samples were treated with 2% trypsin (ab970, Abcam, Cambridge, UK) at 37°C for 15 min and incubated in 3% H_2O_2 solution for 15 min to inhibit endogenous peroxidase activity. Then, the sections were incubated with anti-POMC (PAB 17131, Novus Biologicals, Littleton, CO, USA), polyclonal antibody in a 1/100 dilution for 18 h at +4°C. They were then given three additional 5-min washes in PBS, followed by the incubation with biotinylated Ig G and administration of streptavidin peroxidase (Histostain Plus kit Zymed 87-9999; Zymed, San Francisco, CA, USA). After washing the secondary antibody with

PBS, three times, for 5 min, the sections were stained with DAB substrate system containing diaminobenzidine (DAB, K007, DBS, Pleasanton, CA, USA) to detect the immunoreactivity, and then stained with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany) for counterstaining. For positive and negative control purposes, a mouse suprarenal gland was applied the same procedure; however, normal IgG in place of primary antibody was used as a negative control. They were covered with a mounting medium (01730 Surgipath, Cambridge, UK) and observed with a light microscopy (Olympus BX-40, Tokyo, Japan).

Immunostaining for POMC expression in the bladder samples were evaluated semi-quantitatively using HSCORE analysis according to the method published by McCarty et al.[7] The immunostaining intensities were categorized by the following scores: 0 (no staining), 1 (weak, but detectable, staining), 2 (moderate staining), and 3 (intense staining). A HSCORE value was derived for each specimen by calculating the sum of the percentage of cells for urothelium of both bladder that stained at each intensity category, multiplied by its respective score, using the formula $H\text{-}score = \sum Pi\ (i+1)$, where i is the intensity of staining with a value of 1, 2, or 3 corresponding to weak, moderate, or strong staining, respectively, and Pi is the percentage of stained cells for each intensity, in the range of 0–100%. For each slide, five different fields were evaluated microscopically at 200× magnification. HSCORE evaluation was performed independently by at least two investigators (KO, TO, MT) blinded to the source of the samples as well as to each other's results; the average score of both was then used.

Statistical analysis

For the statistical analysis we used the software SPSS 19.0. The numerical variables are given with mean, standard deviation, median, and minimum and maximum values. Kruskal–Wallis tests were used for comparisons in between the four groups for parametric variables, and the Tukey test was used for post-hoc tests. Additionally, we used the Kruskal–Wallis test for comparisons in between the four groups for non-parametric variables and the Mann–Whitney U test with Bonferronni correction for post-hoc tests. Statistical comparisons with a P value below 0.05/4 = 0.013 are assumed as statistically significant.

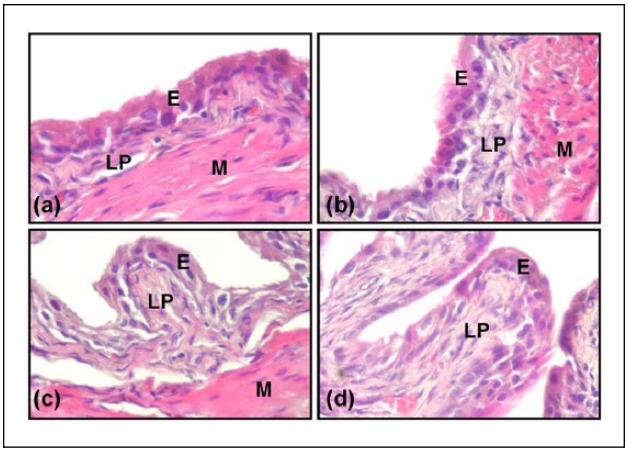

Figure 1. The bladder is lined by the transitional epithelium (urothelium) (E) with its lamina propria (LP), a thick muscularis layer (M). There are no differences between the groups in the H&E staining. (a) Group 1, (b) Group 2, (c) Group 3, (d) Group 4 (original magnification: 400×).

Results

We observed in the H&E staining bladder slides of control group, transitional epithelium was line the bladder mucosa. The epithelium has six and eight layer cells and consists of basal, intermediate, and superficial cell layers. Beneath the epithelium, there is a discontinuous muscularis mucosa, which arranged irregular muscle fibers. In lamina propria of the control group, a few small mucus secreting glands were seen. The muscularis layer is arranged as three layers: the inner and outer longitudinal layers, and the central layer circularly arranged. We show that mice, subjected to radiation exposure, developed mild inflammatory response in bladder tissue characterized by submucosal edema (Groups 2, 3, and 4) (Figures 1a–d).

We investigate the change of the POMC expression of urothelium of the bladder samples after radiation exposure in groups one to four. The immunohistochemical investigation of the urothelium of bladder tissue samples for POMC shows different staining levels. We observed very weak staining for Group1 (96.7 ± 7.68) but the stained cells were localized in the superficial and intermediate layer of urothelium (Figure 2a). Also the staining intensities were low in number in both Group 2 (88.3 ± 8.04) and Group 3 (85.10 ± 10.9) (Figure 2b, c). A strong POMC immunoreactivity

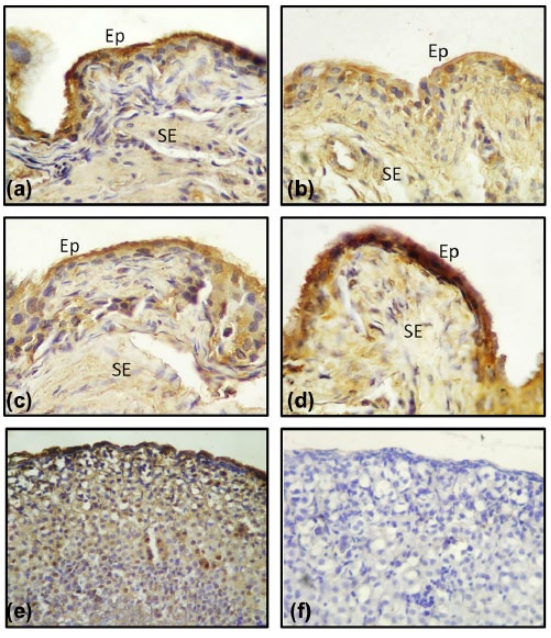

Figure 2. Immunohistochemical analysis of POMC expression was seen in the urothelium of bladder. The moderate POMC expression was seen in urothelium of group 1 (a), group 2, and group 3 (c). In contrast to this, in group 4, strong POMC immunoreactivity was seen (d). Positive control: Mouse suprarenal gland was analyzed by immunohistochemical methods using POMC antibody for positive control purpose (e) and without antibody for negative control purpose (f). E, epithelium; LA, Lamina propria (original magnification: 400×).

was observed within all layers of the urothelium of Group 4 (113.0 ± 12.8), shown in Figure 2d. All comparisons for H-scores between Group 4 and the other groups (Groups 1, 2, and 3) were statistically significant (Tables 1 and 2).

Discussion

We investigated whether POMC expression contributes to acute radiation effects in the rat urinary bladder and used the radiation dose of 10 Gy, as used in previous reports.[8] The radiation dose calls as intermediate dose, between low doses typically in the range of 0.015–2 Gy and high doses in the range of 30–45 Gy.[2]

Although radiotherapy is widely used in the treatment of cancer, the response of tissues to radiation is variable.[9] It was reported that irradiation of the urinary bladder results in morphological changes, such as mucosal edema and hyperemia. The findings are seen in the acute phase and then

Table 1. POMC expression values among groups of duration of radiation exposure.

	Bladder
n	40
Median	94,0000
Min–max	70–131
Chi-Square	17,945*
df	3
P	0.000

*Four cells (50.0%) have expected frequencies less than 5. The minimum expected cell frequency is 4.8.

the reduction in the bladder storage capacity and a consequential increase in micturition frequency.

It is well known that in tissues with high proliferation rates (with cell turnover times ranging from a few days to several weeks), such as the epithelium of the intestine, the oral mucosa or the epidermis, a progressive decrease in cell numbers during radiotherapy occurs due to the continuing loss of cells. The mechanism of radiation damage in the urinary bladder is clearly different from tissues with more rapid turnover times and we found no morphological differences and mitosis on urothelium of bladder between groups, and consider it possible that the normal appearance may be related with the low mitosis index. It is known that under normal unstimulated conditions, the bladder epithelium has an extremely slow cell turnover rate.[11] However, urothelial proliferation is dramatically upregulated in response to injury, resulting in complete restoration of differentiated superficial cells within 7 days.[12]

In this study, we observed for the first time that POMC immunoreactivity was in the urothelium of control group bladder, and we consider it possible that POMC peptides may have constitutively expressed in the bladder, as in the epidermis. Primary keratinocytes constitutively express the full-length POMC mRNA[13] as well as normal human keratinocytes and a keratinocyte cell line produce POMC-derived peptides such as α-MSH and ACTH.[14] The POMC expression in the bladder may be related with cell differentiation, because mitoses were never found in the original superficial cells. Slominski et al. showed that in the epidermis, POMC derived α-MSH was strongly expressed in differentiated cells of supra basal layers, whereas only weakly expressed in basal layer and stratum corneum.[15] Kreft et al. reported that

Table 2. Post-hoc comparisons* of the groups of radiation exposure in regard to cell counts.

		Control	Group 1	Group 2	Group 3	*P*
Bladder	> Median	5	1	3	10	(0 = 1 = 2) <3
	<= Median	5	9	7	0	

*Mann–Whitney U pair comparisons with critical *P* value = 0.0125.

the urothelial cell divisions during the wound-healing process confirmed mitoses only in the basal cells.[16]

We also found that the POMC expression continued in radiation groups 2 and 3, and there were no statistical differences from control group and consider that 10 Gy radiation appears not to seriously damage the bladder. Jaal and Dorr reported that 20 Gy radiation dose causes a decrease in the number of cells of the urothelium in the early (0–31 days) and late stages (90–120 days).[3] Antonakopoulos et al. reported morphological changes in rat urothelium as late as 6 months after single dose irradiation with 20 Gy.[10] Reitan reported that urothelium has low compensatory capacity irradiation, because of a recovery period of about 3 months following 10 Gy and approximately a 9-month period following 20 Gy.[17]

The mechanism of radiation damage in the urinary bladder is clearly different from tissues with more rapid turnover times. Stewart et al. showed that the urothelium of mice at 2 weeks after 30 Gy was intact, with large superficial cells lining the luminal surface of the bladder, whereas the first histological changes were detectable at about 5 months.[18] Cell depletion in the urothelium and the loss of the umbrella cells do not reflect gross tissue breakdown, as it is induced by irradiation in other, more rapidly proliferating tissues.

We observed that a single dose of 10 Gy radiation could activate the POMC expression in urothelium of bladder on day 7. It is important for bladder urothelium to recover from epithelial injury in a short time,[19] because a composition of the urothelium enables the maintenance of the most impermeable barrier in the body, and protects the underlying tissues from toxic urinary substances. Tight junctions between urothelial cells are located on the apical membrane of big hexagonal superficial umbrella cells and a disruption of the tight junction leads to leakage of the urine components into the underlying tissues. Stelwagen et al. reported that in the mammary epithelium, the tight junction permeability decreases milk secretion, and demonstrated that elevated plasma cortisol reduced mammary tight junction leakiness.[20] In addition, in the bladder, administration of recombinant human keratinocyte growth factor, which is known to increase urothelial cell proliferation,[21] ameliorated both early and late radiation-induced urinary bladder dysfunction in mice.[22]

It is known that POMC's role in skin is pigmentation and peripheral modulation of stress and inflammatory responses.[23] We suggested that the increase of POMC in all layers of bladder on day 7 may be related with inflammation response as with skin. POMC peptides are produced locally in the skin and are regulated by inflammatory cells, as well as by autocrine mechanisms.[6] Slominski et al. reported recently that a stress response system may exist in the skin mediated by the neuroendocrine system and modulated by the immune system, with the CRH-POMC loop playing an essential role. IL-1, which forms an important part of the inflammatory response, increases the production of POMC mRNAs, ACTH, and MSH peptides in cultured epidermal keratinocytes[24] and melanocytes.[25] We similarly suggest that the immune system could play a dominant regulatory role in POMC peptide production in the bladder, as with the skin. In the bladder, the major pro-inflammatory cytokines, interleukin (IL)-1, tumor necrosis factor (TNF)-α, IL-6, and IL-8 are released from granulocytes, macrophages, monocytes, and other immunoregulatory cells during inflammatory reactions. Chuang et al. transferred the POMC gene in acetic-acid-induced bladder hyperactivity using the gene gun, and reported that increased bladder expression of endorphin can suppress nociceptive responses induced by bladder irritation.[26]

Our study demonstrated that irradiation results in increased expression of the POMC with the time course that parallels the radiation injury. Therefore, it is possible that the differentiation and restoration of the bladder urothelium is based on the overexpression of POMC from urothelial cells.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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