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NUCLEAR ACCUMULATION OF PROTEIN P53 AND HISTOLOGICAL CHANGES IN THE RAT MODEL OF UNILATERAL CRYPTORCHIDISM

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ABSTRACT

Introduction: Cryptorchidism is accepted as a substantial risk factor for the subsequent development of testis cancer. Since p53 is highly expressed in testicular tumors, this study was undertaken to investigate the abnormal expression of p53 protein and histological changes in a rat model of unilateral cryptorchidism.

Material and Methods: Prepubertal rats were rendered mechanically rendered unilaterally cryptorchid on 15th day after the birth. Subsequently, testes were harvested from experimental and sham operated rats at two, four and six months for immunohistochemical and histological studies. Contralateral testes in the experimental groups served as the controls.

Results: There were no histological abnormalities observed in the testes of both sham operated and control groups. However, experimentally cryptorchid testes were smaller and accompanied by a prominent change in color. A significant decrease in testicular volume in the cryptorchid group was noted ($p < 0.05$). Seminiferous tubular atrophy, basement membrane thickening, germ cell loss and spermatogenic arrest were progressively more evident with time, being highest at the sixth month. Immunohistochemical staining on paraffin sections demonstrated positive nuclear reaction for p53 in 14 (93.3%) out of 15 cryptorchid testes. In contrast, sham operated and control groups were completely devoid of any nuclear accumulation of p53.

Conclusion: In conclusion, this study confirms that intraabdominal cryptorchid testes are significantly impaired due to abnormal localization. Immunohistochemical positivity for the p53 protein in cryptorchid testes suggests a molecular alteration and may indicate an association between cryptorchidism and testicular carcinogenesis.

Key words: testis; cryptorchidism; protein p53; testicular neoplasms; rat
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INTRODUCTION

Cryptorchidism is a common congenital abnormality. The incidence of cryptorchidism in term infants is approximately 3% and it decreases to 0.8% by one year of age (1). Based on various clinical and experimental studies, cryptorchidism is identified as a cause of testicular atrophy and subsequent development of testicular neoplasm (2,3). It is known that 10% of testicular cancer originate from the ipsilateral cryptorchid testis and the incidence of carcinoma in situ in cryptorchid testes was reported as 1.7% (2). Despite a large body of clinical and experimental evidence related to cryptorchidism and its relation with testicular cancer, the mechanism of damage at molecular level resulting in microscopic and macroscopic abnormalities has not been explored in depth.

The p53 gene is a tumor suppressor gene that exerts a negative regulatory effect on cell growth (4). Different studies have demonstrated a significant correlation between the functional inactivation of the p53 tumor suppressor gene and tumor grade and stage. It is also found to be a predictor of tumor behavior with a poor prognosis in tumors such as breast cancer,

colon cancer and other gastrointestinal malignancies (5,6). The wild-type p53 gene product is undetectable by immunohistochemical methods due to its short half-life and the low amount of p53 present (7). However, mutation of p53 results in stabilization of the protein with a prolonged half-life (8,9), and makes it detectable by immunohistochemistry (7). Abnormal p53 expression has been implicated as a valuable tumor marker for some neoplasms including bladder cancer (10).

This study was undertaken to define the status of nuclear accumulation of p53 indicating an aberrant protein and associated histological change in an animal model.

MATERIAL AND METHODS

Animals

Fifteen-days-old Wistar Albino Rats (20 - 30 gr.) were housed at the Laboratory Animal Research Center at Marmara University. Experimental design and treatment of these animals was approved by the Animal Care Committee of our institution. Animals were caged under controlled lighting (12 hours light, 12 hours dark) and temperature (24^{o}C). Food and water were provided ad libitum. The rat testes were divided into three groups: 1)- mechanically produced left-sided cryptorchid testes (n = 15 testes); 2)- sham-operated testes (n = 6 testes); and 3)- the right testes of the sham operated and cryptorchid group were used as control (n = 15 + 6 = 21 testes). Rats were rendered cryptorchid by obstructing left internal inguinal canal with a 4-0 vicryl suture. Five rats from cryptorchid group and two rats from sham-operated group were sacrificed after bilateral orchiectomy, in the second, fourth and sixth month after first operation, respectively. After measurement the three dimensions of each testis, the testicular volume was calculated by using the "Ellipsoid formula". Testes were then placed in Bouin's fixative solution for 6 hours at room temperature, embedded in paraffin. Sections of 5-micrometer thickness were obtained using a standard rotary microtome and fixed onto silanized slides for immunohistochemical and histological studies.

Surgical Procedure

After induction of anesthesia with an intraperitoneal injection of ketamine 100 mg/kg plus xylazine 1 mg/kg, rats were placed in a supine position and a vertical midline incision of about 2 cm was made in the abdomen just above the base of the penis. At this age, rat testes are still intraabdominal. Through this approach intraabdominal, testes were identified on either side. The left internal inguinal canal was closed with 4/0 vicryl suture to prevent left testicular descend.

Sham-operated rats (n = 6) were subject to a similar vertical midline incision of about 2 cm in the abdomen just above the base of the penis. Testes were identified on either side. The left-sided testis and left internal inguinal ring were touched with a cotton swab as in preparation for vicryl suture placement. Abdomen was closed without further intervention.

The effectiveness of the surgical procedure was evaluated by confirming the intra-abdominal location of the experimental cryptorchid testis and by verifying that the inguinal canal was closed.

p53 Immunohistochemistry

The antibody used in this study was a mouse monoclonal antibody (pAb 1801) against p53 mutant type. In preparation for immunohistochemical procedures, the tissue was deparafined, rehydrated, and placed in a solution of hydrogen peroxide for 10 min. An antigen retrieval method was used. Sections were placed in 10 ml of citrate based retrieval solution, mixed with 30 ml of water, and heated in a microwave for a total of 15 min at room temperature at 100% power. Tissue slides were cooled for 20 min at room temperature, rinsed with water and phosphate-buffered saline, and immersed into a blocking serum (10% Rabbit non-immune serum, Zymed Laboratories Inc., South San Francisco, CA) for 1 h. at room temperature. The antibody was then incubated with the tissue sections overnight at 4^{o}C. Tissue sections were incubated for 30 min at room temperature with Biotinylated goat anti-mouse antibody (Zymed Laboratories Inc, South San Francisco, CA). Color was developed by diaminobenzidine tetrahydrochloride solution (Zymed Laboratories Inc, South San Francisco, CA) and sections were counterstained with he-

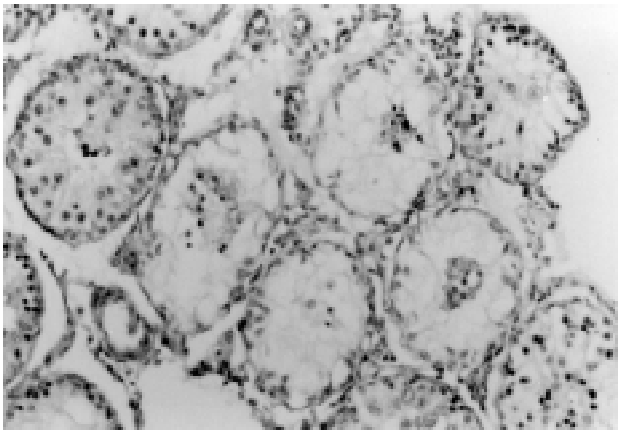

Figure 1 *- Abnormal p53 nuclear accumulation observed in a cryptorchid rat testis (Immunolabeling, X100)*

matoxylin. Immunohistochemical reaction was considered positive only in cases of nuclear staining. P53 nuclear positivity was expressed in "%" after counting a total of 400 cells from each slide by using the formula; p53 (%) = 100 x (p53 positive cell number/ 400). Two investigators reviewed all slides independently.

Differences between the groups were tested for significance by "Dunn`s Multiple Comparisons Test" and "Mann-Whitney Test".

RESULTS

p53 Expression

Cryptorchidism group - p53 expression: abnormal p53 nuclear accumulation was detected by immunohistochemistry in 14 of 15 cryptorchid testes (93.3%) (Figure-1). Ratio of nuclear p53 staining was 0.32 ± 0.28%, 0.41 ± 0.3%, and 0.26 ± 0.06% at second, fourth, and sixth months, respectively (Table-1).

Control group - p53 expression: no abnormal nuclear accumulation was detected in any one of the 21 testes.

Sham-operated group -p53 expression: immunohistochemistry did not reveal any p53 expression in sham-operated animals.

The p53 nuclear staining of cryptorchid testes was considered statistically significant. There was no statistical significance ($p > 0.05$) between the nuclear p53 staining of the 2, 4 and 6-month cryptorchid testes (Table-1).

Effect of Cryptorchidism on Testicular Volume

No significant differences in testicular volume were noted between the left-sided cryptorchid sub-groups of testes at month 2, 4 and 6 of operation ($p > 0.05$, Table-2). A significant decrease ($p < 0.05$) in testicular volume ($cm^3 \pm SD$) was seen between the left-sided cryptorchid testes and controls at each examination group. In fact, there was a significant increase ($p < 0.05$) in the volume ($cm^3 \pm SD$) of testes of the control group with time. Table-2 illustrates the comparison of testicular volume changes between groups.

Effect of Cryptorchidism on Testicular Histology

The histologic findings of the control and sham-operated testes (n = 15, n = 12 respectively) were normal. In contrast to sham-operated and control groups, undescended testis group was histologically characterized by early maturation arrest, decrease in seminiferous tubule diameter and thickening of basal membrane. These abnormal histological changes were first observed at 2 months and were

Table 1 *- Comparison of the ratio (%) of nuclear p53 staining among cryptorchid, control, and sham operated testes related to time (mean ± SD).*

Groups	2nd Month	4th Month	6th Month
Cryptorchid (No. = 5)	1.2 ± 0.77	1.3 ± 1.13	2.1 ± 1.75
Control (No. = 5)	0	0	0
Sham-operated, right testes (No. = 2)	0	0	0
Sham-operated, left testes (No. = 2)	0	0	0

Table 2 - *Comparison of testicular volume (cm^3) changes among cryptorchid, control, and sham operated testes related to time (mean ± SD).*

Groups	2nd Month	4th Month	6th Month
Cryptorchid (No. = 5)	0.32 ± 0.28	0.41 ± 0.3	0.26 ± 0.06
Control (No. = 5)	1.33 ± 0.10	1.46 ± 0.16	2.08 ± 0.12
Sham-operated, right side (No. = 2)	1.55 ± 0.24	1.7 ± 0.08	1.59 ± 0.05
Sham-operated, left side (No. = 2)	1.38 ± 0.17	1.56 ± 0.09	1.37 ± 0.02

more prominent at 6 months. The most distinctive histological abnormality seen at 6 months was the "Sertoli cell only syndrome" in some areas of testicular tissue (Figure-2).

DISCUSSION

Testis cancer is one of the commonest cancers in young men with a substantial increase in incidence in many white populations (11). Contemporarily, cryptorchidism is the only well-established risk factor in etiology. The risk of a cryptorchid testis developing cancer has been estimated up to 40 times higher than that of a scrotal testis (12).

The objective of this study was to observe whether there was any correlation between undescended testes and abnormal p53 protein expression and the abnormal testicular histological findings caused by intraabdominal localization of testes. In this animal model of cryptorchidism, testicular development was arrested, and spermatogenesis was impaired dramatically as well. Both macroscopic and microscopic changes in the left testis were readily apparent at two months and became more obvious in the sixth month. Penson et al. also recognized similar changes in testicular volume in their animal model (13). It is interesting that we noted an increase in testicular volume in contralateral testes of the cryptorchid group, particularly in the sixth month in comparison to testes of the sham-operated rats. This may indicate some sort of compensatory changes in opposite testes just like compensatory renal hypertrophy. Microscopic findings of cryptorchid testes were typical. There was prominent and progressive decrease in the diameters of seminiferous tubules of cryptorchid testes when compared to the contralateral and sham-operated testes. These changes were observed as early as in the second month, and progressively became prominent in the fourth and sixth months. The thickness of basal membrane was increased at fourth month, and it was more evident by six months. In all cryptorchid testes spermatogenetic arrest was observed. Mature spermatides could be seen in the second month with a lesser degree of arrest. By the forth month spermatogenetic arrest was at the stage of spermatocytes, and this was more obvious by the sixth month. Subsequently, severe spermatogenetic arrest was seen in undescended testes, which would correspond to "Sertoli cell only syndrome". However, there was no change in the number of the Leydig cells, which constitutes the major part of interstitium. Penson et al also reported

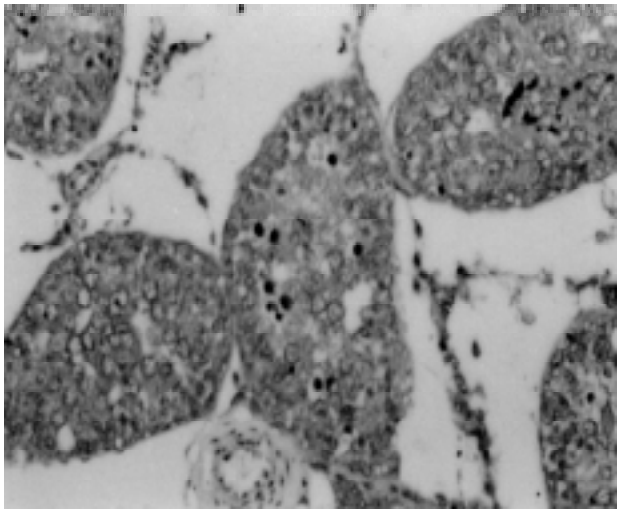

Figure 2 - *Sertoli cell only syndrome observed as patchy areas in a cryptorchid testis at six months. Note the apparent decrease in seminiferous tubule diameter and thickening of the basal membrane (HE, X100).*

no significant increase in the number of Leydig cells. In literature several studies propose that the histological abnormalities observed in the cryptorchid testes are due to a primary genetic alteration (14,15). They postulate similar histological changes in the contralateral testes. Others propose an autoimmune etiology as the causative factor in the development of these histological changes in cryptorchid testes. However, in our study histological changes were only observed in the cryptorchid testes. Contralateral testes showed normal maturation, both macroscopically and microscopically. This observation suggested that abnormal localization of the testes was the principal factor for these histological alterations. Exposure to higher temperatures due to abnormal position was accused as the cause of histological and spermatogenetic defects in several studies (13, 16,17).

In contrast to histological changes, which were extensively studied, any alteration at the molecular level in the cryptorchid testes has not been largely analyzed. Moreover, the association between cryptorchid testes and testis cancer suggests a possible modification at the genetic level in the cryptorchid testes, since a significantly higher incidence of testis cancer has been recognized in cryptorchid testes (2,18-21). Increased expression of p53 in germ cell tumors has been reported in several studies (2,22,23). Bartkova et al. found nuclear p53 staining in 84% of the testis cancers (20). The current study is valuable in terms of anticipating a possible association between p53 expression and cryptorchidism. According to our results, p53 expression was seen only in cryptorchid testes. Almost all of the cryptorchid testes (14 cases out of 15) showed some degree of positive staining for p53. Nuclear staining was observed between 0.25% and 5% with the mean ratio of 1.53% of the nuclei examined in cryptorchid testes. Although this was not statistically significant, p53 expression was gradually increased with time being highest in the sixth month. This observation suggests that there is also a molecular alteration beside histological changes due to abnormal localization of the testes. Nuclear accumulation of p53 in cryptorchid testes may cause a defect at the cell cycle regulation in these cells, which in turn may result in initiation of carcinogenesis. In a similar experimental model, Socher et al. rendered male adult CD-1 mice unilaterally cryptorchid by a comparable surgical method. They evaluated p53 expression by Western blot analysis (24). Altered expression of p53 protein in the cryptorchid testis was seen beginning on day 7. They also observed that the weights of the cryptorchid testes decreased by approximately 40%. Histological evidence of germ cell loss was also noted. They proposed that the p53 as an inducer of apoptotic cell death has a significant role in temperature-mediated germ cell loss. However, it would theoretically be also possible that the presence of p53 expression in the cryptorchid testis might be due to the undescensus itself, as the associated growth retardation, seminiferous atrophy, etc. Similarly, there are several theories including endocrine dysfunction, interference with blood supply, gonadal dysgenesis, etc. on neoplastic process in cryptorchid testis leading to testis cancer (25). Nevertheless, the results of the current study indicating p53 expression in cryptorchid testis open new avenues in the research on cryptorchidism at molecular level.

CONCLUSION

The results of this experimental study demonstrate both microscopic and macroscopic changes in cryptorchid testes. The documentation of nuclear p53 expression in cryptorchid testes also suggests also an alteration at the genetic level. In this regard, observation of p53 expression in cryptorchid testis proposes a possible role of p53 in the development of testis cancer in cryptorchid testes. However, further experimental and clinical studies are required to reach more definite statements.

REFERENCES

1. Kogan SJ: Cryptorchidism. In: Kelalis PP, King LR, Belman AB (eds.). Clinical and Pediatric Urology. Philadelphia, WB Saunders Co., pp 1050-1083, 1985.

2. Rajfer J: Congenital Anomalies of the Testis and Scrotum. In: Walsh PC, Retik AB, Vaughan ED, Wein JA (eds.). Campbell's Urology. Philadelphia, WB Saunders Co., pp. 2172-2192, 1998.
3. Beard CM, Benson RC Jr, Kelalis PP, Elveback LR: Incidence of malignant testicular tumors in the population of Rochestes, Minnesota. Mayo Clinic Proc, 52: 8-10, 1977.
4. Sidransky D, von Esehenbach A, Tsai YC, Jones P, Summerhayes I: Identification of p53 gene mutations in bladder cancers and urine samples. Science, 254: 706-709, 1991.
5. Starzynska T, Bromley M, Ghosh A, Stern PL: Prognostic significance of p53 expression in gastric and colorectal carcinoma. Br J Cancer, 66: 558-562, 1992.
6. Thor AD, Moore DH, Edgerton SM, kawasaki ES: Accummulation of p53 tumor supressor gene protein. An independent marker of prognosis in breast cancers. J Nat Cancer Inst, 84: 845-855, 1992.
7. Varley JM, Brammer WJ, Lane DP, Swallow JE, Dolon C, Walker RA: Loss of chromosome 17p13 sequences and mutation of p53 in human breast carcinomas. Oncogene, 6: 413-421, 1991.
8. Crawford LV, Gurney EG, Goolfellow P, Taylor PJ: Detection of a common feature in several human tumor cell lines. A 53.000 dalton protein. Proc Natl Acad Sci, 78: 41-45, 1981.
9. Klein N, Vignaud JM, Sadmi M, Plenat F, Borelly J: Squamous metaplasia expression of protooncogene and p53 in lung cancer patients. Lab Invest, 68: 26-32, 1993.
10. Wright C, Mellon K, Jonston P, Lane DP, Harris AL, Horne CH, Neal DE: Expression of mutant p53, c-erb B-2, and the epidermal growth factor receptor in transional cell carcinoma of the human urinary bladder. Brit J Cancer, 63: 967-977, 1991.
11. Swerdlow AJ, Huttly SRA, Smith PG: Testicular cancer and antecedent diseases. Br J Cancer, 55: 97-103, 1987.
12. Chilvers C, Dudley NE, Gough MH, Jackson MB, Pike MC: Undescended testis: The effect of treatment on subsequent risk of subfertility and malignancy. J Ped Surg, 21: 691-696, 1989.
13. Penson DF, Lugg JA, Coyne C, Sadeghi F, Freedman AL, Gonzales CNF, Raijfer J: Effect of cryptorchidism on testicular histology in a naturally cryptorchid animal model. J Urol, 158: 1978-1982, 1997.
14. Scott LS: Delayed treatment of cryptorchidism with subsequent infertility. Fertil Steril, 18: 782-787, 1967.
15. Shirai M, Matsushita S, Kagayama M, Ichijo S, Takeuchi M: Histological changes of the scrotal testis in unilateral cryptorchidism. Tohoku J Exp Med, 90: 363-373, 1996.
16. Bedford JM: Effects of elevated temperature on the epididymis and testis: experimental studies. Adv Exp Med Biol, 7: 19-32, 1991.
17. Loughlin KR, Manson K, Foreman R, Schwartz B, Heuttner P: The effect of intermittent scrotal hyperthermia pn the Sprague-dawley rat testicule. Adv Exp Med Biol, 7: 183-185, 1991.
18. Henderson BE, Benton B, Jing J, Yu MC, Pike MC: Risk factors for cancer of the testis in young men. Int J Cancer, 23: 598-602, 1979.
19. Depue RH: Maternal and gestaional factors affecting the risk of cryptorchidism and inguinal hernia. In J Epidemiol, 13: 311-318, 1984.
20. Gershman ST, Stolley PD: A case control study of testicular cancer using Connecticut tumor registry data. Int J Epidemiol, 17: 738-742, 1988.
21. Benson RC, Beard CM, Kelalis PP, Kurland LT: Malignant potential of the cryptorchid testis. Mayo Clinic Proc, 66: 372-378, 1991.
22. Bartkova J, Bartek J, Lukas J, Výjtesek B: p53 protein alteration in human testicular cancer including pre-invasive intratubular germ cell neoplasia. Int J Cancer, 49: 196-202, 1991.
23. Lewis DJ, Sesterhenn IA, McCarthy WF, Moul JW: Immunohistochemical expression of p53 tumor suppressior gene in adult germ cell testis tumors: clinical corrrelation in stage I disease. J Urol, 152: 418-423, 1994.
24. Socher SA, Yin Y, Dewolf WC, Morgentaler A: Temperature-mediated germ cell loss in the testis is associated with altered expression of the cell-cycle regulator p53. J Urol, 157: 1986-1989, 1997.

25. Richie JP: Neoplasms of the Testis. In: Walsh PC, Retik AB, Vaughan ED, Wein JA (eds.). Campbell's Urology. Philadelphia, WB Saunders Co., pp. 2411-2452, 1998.

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EDITORIAL COMMENT

Individuals born with an undescended testis have approximately a 40-fold increased incidence of testicular malignancy. The background of the increased risk for cancer is largely unknown. The present manuscript is, therefore, of great value since it suggests a genetic molecular pathogenesis, and quite convincingly so. This paper opens new avenues in the research around cryptorchidism. Subsequent research has to focus on the association between the abnormal expression of the p53 protein in undescended testes and the pathogenesis of cryptorchidism itself.

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