The dynamics of the imprinted H19 gene expression in the mouse model of bladder carcinoma induced by N-butyl-N-(4-hydroxybutyl)nitrosamine

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The imprinted H19 gene product is an oncofetal RNA molecule in humans. It is expressed in fetal bladder, down-regulated postnatally and is re-expressed in human bladder carcinoma. This study was designed to investigate the dynamics of the expression of H19 in the mouse bladder carcinoma induced by N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) and its relation to stages of neoplastic transformation. BBN was administered to mice in the drinking water for 26–28 weeks. The bladders were removed at 5–11 weeks after administration. A total of ten bladders from the mice in each group were processed for hybridization. In situ hybridization was performed using an ~400 base 35 S-labeled antisense probe. Following BBN administration expression of H19 first appeared after 5 weeks in the lamina propria adjacent to the basement membrane, concomitant with mucosal hyperplasia. At 11 weeks focal expression was noted in epithelial cells. Invasive carcinomas, of the transitional and squamous sub-types, were seen after 20 weeks and more of BBN administration. At this stage H19 expression was observed in scattered tumor cells, in the connective tissue stroma of the tumor and in the lamina propria underlying the remaining hyperplastic/dysplastic mucosa. Abundant expression of H19 was evident in fetal bladder but was absent in normal adult bladder. We conclude that, similar to humans, the H19 gene product is an oncofetal RNA molecule in the experimental mouse model of bladder carcinoma. In this model H19 is expressed in the connective tissue of the lamina propria prior to its expression in epithelial cells, concurrent with preneoplastic changes in the transitional epithelium of the bladder.

Introduction

Bladder carcinoma is one of the most prevalent cancers in the industrialized world, and the incidence is higher in urban than in rural environments. Exposure to industrial carcinogens is considered a major pathogenic mechanism. Workers at risk for developing bladder cancer are those of the aniline dye industry as well as rubber workers, leather processors, painters, organic chemical producers, textile workers and hairdressers (1,2). The common denominator of these industries is exposure to aryl amines. These organic chemicals are excreted from the body through the kidneys and their concentration in the urine may cause chemical carcinogenesis. Another important risk factor is cigarette smoking, which is related to the presence of aromatic amines, nitrosamines or both in the tar component.

An animal model of chemical carcinogenesis of the urinary bladder using N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) was first developed in rats by Ito et al. (3) and later in mice by Hirose et al. (4). Following administration of BBN rats develop papillary non-invasive tumors of the urinary bladder, whereas mice developed invasive carcinoma preceded by preneoplastic mucosal changes as described by Ohtani et al. (5). These models may represent the two pathways of bladder carcinoma in humans: one is a superficial papillary, often recurrent tumor, and the other is solid invasive cancer (6).

In recent years we have studied the expression of the H19 gene in human bladder carcinoma (7–10). H19 is an imprinted gene in mice (11) and humans (12,13). Imprinted genes—genes which are expressed from one allele depending on the gamete-of-origin—are implicated in playing an important role in tumorigenesis of certain human neoplasms (14,15). H19 is expressed from the maternal allele and its product is an untranslated RNA molecule (16) with regulatory functions. Like other imprinted genes it is abundantly expressed in fetal life and down-regulated in most tissues postnatally (17,18).

We have proposed that H19 RNA is an oncofetal gene product because it is re-expressed in tumors arising from tissues which express the gene in fetal life (7,10,19). Human bladder cancer is a prototype of tumors which abundantly express H19, arising from the transitional epithelium of the urinary tract which expresses H19 in fetal life.

We used the BBN-induced bladder carcinoma mouse model in order to correlate H19 expression, as demonstrated by in situ hybridization, to the sequential stages of tumor progression. We found that following exposure to the carcinogen the H19 gene was first expressed in connective tissue of the submucosa concomitantly with epithelial preneoplastic hyperplasia, and was later expressed in epithelial cells during tumor evolution, almost exclusively in invasive cancer.

Materials and methods

C3H/He female mice 6 weeks old (Harlan Laboratories, Jerusalem, Israel) were placed in plastic cages in groups of five and were given food and water ad libitum. BBN (Tokyo Kasai, Tokyo, Japan) was dissolved in tap water to a concentration of 0.05% and supplied to group 1 ad libitum. Group 2, the control group, was given tap water throughout the experiment.

Mice from each group were killed after 5, 10, 20 and 26 weeks, and on repeat experiment the animals were killed at 5, 11, 20 and 28 weeks. A total of four animals were killed at 5 weeks, eight at 10–11 weeks, 11 at 20 weeks and 14 animals at 26–28 weeks. The urinary bladders were immediately removed, their lumen was rinsed with 4% buffered formalin and the whole bladder was placed in plastic cassettes for in situ hybridization. A total of ten bladders from the mice in the control group (Group 2), which were killed in the same time periods, were also processed for in situ hybridization.

In situ hybridization was performed using an ~400 base 35 S-labeled antisense RNA probe, generated by in vitro transcription of plasmid subclone of murine H19 (kindly provided by S.M.Tilghman from Princeton University, Princeton, NJ) by T3 polymerase. A sense RNA probe generated by T7 polymerase was
used for control. The procedure of the in situ hybridization was carried out as previously described (10).

Fetal bladder was obtained from mouse fetuses on the 17th gestational day. The histopathological terminology used in this study was the same as used for human tumors (20), with minor modifications according to the classifications of bladder carcinoma in rodents (21,22).

Results

The development of BBN-induced urinary bladder carcinoma in mice was time dependent and occurred following precancerous epithelial changes, essentially similar to the changes previously described by Ohtani et al. (5). H19 expression was first noted in the connective tissue of the superficial submucosa (lamina propria) and at a later stage in the premalignant and malignant epithelium.

Fetal bladder

Prominent H19 expression was noted in the submucosa as well as in the transitional epithelium of the mucosa and to a lesser extent in the connective tissue between bundles of smooth muscle in the muscular layer (Figure 1A and B).

Control group—normal adult bladder

A total of ten urinary bladders of the control groups of mice (Group 2), which had tap water without carcinogen for drinking, were examined sequentially during the experiment. In all the animals the urinary bladder mucosa contained no more than three layers of cells, the most superficial sometimes being flattened (also designated umbrella cells). In none of the bladders was H19 expression noted in any of the tissular elements of the bladder wall (Figure 1C and D).

BBN administration for 5 weeks

Epithelial changes were noted in the bladder mucosa of all four mice killed at 5 weeks of BBN administration. These included mild to moderate hyperplasia, with an increase of up to eight cell layers, cellular atypia and increased mitotic activity at the basal layer and in more superficial layers. These changes correspond to dysplasia in human bladder carcinoma (20).

In the submucosa increased cellularity with neovascularization (granulation tissue) and a mixed inflammatory cell infiltrate were noted. Granulocytes focally invaded the overlying epithelium. Large lymphoid aggregates were scattered in the submucosa and muscularis.

Expression of H19 was observed in the superficial submucosa (lamina propria), just below the basement membrane, in three out of four mice (Figure 1E and F). Expression of H19 was not noted in epithelial cells at this stage. No expression of H19 was noted in the inflammatory infiltrate in the submucosa.

BBN administration for 10–11 weeks

The bladders of eight animals were examined after 10–11 weeks of BBN administration. Diffuse hyperplasia of the bladder mucosa, up to 10–12 layers, was noted in seven animals, and only focal hyperplasia in the remaining mouse. Some loss in cell polarization as well as increased mitotic activity in the basal and, sometimes, upper cell layers were also observed.

The changes in the submucosa were similar to those present at 5 weeks, only somewhat more pronounced. These included granulation tissue and mixed inflammatory infiltrate in the submucosa, with focal granulocytic infiltration of the mucosa and numerous large lymphoid aggregates in the submucosa and muscular layers.

Expression of H19 was noted focally in the lamina propria adjacent to the basement membrane in all but one animal. The bladder without H19 expression was the one which manifested only mild focal hyperplastic changes of the mucosa. In one mouse cells of the connective tissue deeper in the submucosa also expressed H19.

Expression of H19 in epithelial cells of the hyperplastic mucosa was observed in one out of eight mice. The expression was localized mainly to cells of the basal layer and focally in the midlayers (Figure 1G and H).

BBN administration for 20 weeks

Invasive carcinoma was present in nine of 11 bladders of mice following the administration of BBN for 20 weeks. This was squamous cell carcinoma in one case, and the rest were transitional cell carcinomas with focal differentiation to non-keratinizing squamous cell carcinoma. Superficial invasion of the submucosa was observed in a third of the animals with invasive cancer (three out of nine), and in the rest deeper invasion into the muscularis and serosa was noted.

In the non-neoplastic mucosa and in mice without invasive cancer severe hyperplasia of the mucosa (10–15 cell layers) along with loss of polarity and increased mitotic activity were present. Nodular hyperplasia with endophytic growth of the hyperplastic epithelium was seen in six of the cases. In one animal squamous metaplasia with keratinization was observed.

The inflammatory reaction in the submucosa and muscular layers was essentially the same as above, but somewhat less extensive.

H19 expression was seen focally in cells of the lamina propria in nine bladders, in scattered epithelial cancer cells in five out of nine animals and in stromal cells of the tumor in one case.

BBN administration for 26–28 weeks

Invasive carcinoma of the bladder was present in 10 out of 14 mice after 26–28 weeks of BBN administration. In some cases more than one focus of carcinomatous transformation was noted. Differentiation to squamous cell carcinoma was present, at least focally, in seven bladders. All tumors invaded the full thickness of the muscular layer and caused extensive destruction of the bladder wall.

Severe hyperplasia of the mucosa, which was 10–15 layers thick, as well as nodular hyperplasia and dysplastic changes (loss of polarity and increased mitotic activity) were present in the remaining non-neoplastic bladder mucosa.

Lymphocytic aggregates were present throughout the bladder wall, but were generally fewer in number due to the extensive infiltration of the muscle by tumor.

H19 expression was noted in epithelial cells in three out of 10 bladders (Figure 1I and J) and in stromal cells of the tumor in six out of 10 cases of carcinoma. H19 expression was also observed in the lamina propria of non-neoplastic mucosa in six out of 14 (in both tumoral stroma and lamina propria in two cases).

Controls

When expression of H19 in the lamina propria of the bladders of the treated animals (Figure 1K and L) was compared with in situ hybridization of a parallel section using a 35S-labeled sense probe, no signal was observed in any of the tissular elements of the latter (Figure 1M).

The results are summarized in Table I.

Discussion

Animal models have been widely used in the study of cancer. They allow investigation in detail of sequential events occurring
Fig. 1. H19 expression in the mouse bladder following BBN administration (in situ hybridization of 35S-labeled antisense of H19 with hematoxylin and eosin as a counter stain). (A and B) Fetal bladder (bright and dark field respectively). Prominent expression is evident in the mucosa and the connective tissue of the submucosa. (C and D) Adult mouse bladder. Only three layers of cells are noted in the mucosa. No expression of H19 is seen (bright and dark field respectively). (E and F) H19 expression following 5 weeks of BBN administration. The mucosa is hyperplastic. Prominent H19 expression is noted in the superficial submucosa (lamina propria) beneath the basement membrane (bright and dark field respectively). (G and H) Following 11 weeks of BBN administration H19 expression is noted in epithelial cells in the hyperplastic and dysplastic mucosa (arrows) as well as in scattered cells in the lamina propria (bright and dark field respectively). (I and J) Invasive urothelial carcinoma is evident at 28 weeks of BBN administration. H19 expression is seen in malignant epithelial cells (bright and dark field respectively). (K, L and M) demonstrate the control. Expression of H19 is noted in the lamina propria (arrow) in (K) and (L) (bright and dark field respectively). Compare with (M) which is the in situ hybridization of 35S-labeled sense of H19 (dark field), where no expression is noted in the same location (arrow).

during cancer evolution, as well as the application of experimental modes of treatment, ranging from chemicals to gene therapy. The three basic approaches to develop an animal model include transgenic mice, transplantation of human tumors or the corresponding cell lines to nude mice and the induction of cancer by viruses and chemicals.

The mouse has been suggested as an ideal model for studying carcinogenesis of the urinary bladder, because spon-
taneous tumors are rare and other epithelial changes, such as hyperplasia and inflammation, also occur infrequently (21). The experimental mouse model of induction of bladder carcinoma by the carcinogen BBN has been widely used previously to study the sequence of events occurring during bladder carcinogenesis (5,21,23). Chemical carcinogenesis simulates the major pathogenetic mechanism known in human bladder carcinoma. Although distant metastases are usually rare in this model due to the death of the animals caused by early bladder obstruction, the first steps of cancer evolution may be studied in detail.

H19 is an imprinted gene expressed from the maternal allele mapped to an imprinted domain on the short arm of the mouse chromosome 7, together with the insulin-2 gene, insulin-like growth factor 2 and Mash-2 (27). The temporal and tissue-specific expression of H19 and Igf-2 are essentially identical in mice and humans (24–26). H19 encodes an untranslated RNA molecule, which probably functions in the regulation of the oppositely imprinted Igf-2 gene, through the competition between their promoters for a common set of enhancers (27,28).

H19 is abundantly expressed in certain tissue elements in fetal life and its level of expression is down-regulated in most adult tissues in mice and humans (17,18). We have shown that in human cancer H19 exhibits oncofetal characteristics, and in neoplastic tissues its level of expression may resemble that of the same fetal tissues at a similar stage of differentiation (10). H19 may also be re-expressed in regeneration, though usually to a lesser extent than in neoplasia.

In the present study we demonstrate that in mice, similar to humans, H19 is abundantly expressed in the fetal urinary bladder, both in the transitional epithelium and in the connective tissue of the submucosa. The expression, as demonstrated by in situ hybridization, is down-regulated in the normal bladder postnatally. In many aspects the stages of tumor evolution in this animal model resemble the events occurring in human bladder cancer, beginning from hyperplasia through dysplasia to invasive cancer, although some differences include the rarity of papillary tumors, which are the most common in humans (21,22).

In this model the initial expression of H19 was found to be concurrent with epithelial hyperplasia, which appeared as soon as following 5 weeks of carcinogen ingestion. The localization of H19 expression at this stage is the connective tissue of the lamina propria underlying the basement membrane of the hyperplastic epithelium. At 11 weeks some expression was observed in epithelial cells, still in a preneoplastic state. Invasive tumors were first noted at the 20th week of this experiment. H19 expression was evident at this stage both in epithelial tumor cells and in the intervening stroma, as well as in the submucosa underlying hyperplastic epithelium adjacent to the tumor.

The stroma, the connective tissue which interposes between malignant cells, is an integral part of solid tumors. We and others have previously shown expression of H19 in the stroma of tumors. H19 is expressed in the stroma of breast cancer more often than in the tumor cells themselves, and is also expressed in the connective tissue in fibrocystic disease of the breast, a benign condition which may precede neoplastic transformation, and includes a major component of stroma (10,29). We have also observed H19 expression in the stroma of metastatic colon carcinoma, in the absence of expression in the epithelial malignant cells (10).

Mesenchymal tissues during embryogenesis and throughout fetal life express H19. The connective tissue in solid tumors manifests a few characteristics similar to regenerating tissue and fetal tissues. For example, the forms of fibronectin present in tumor stroma and in wound healing, are alternatively spliced forms that are normally synthesized during embryonic life and not in adult tissues (30). In our study H19 expression during the first weeks of the experiment was noted underlying the basement membrane of the hyperplastic epithelium rather than in the granulation tissue deeper in the submucosa. This may point toward the close relationship of the expression of H19 in the connective tissue with the preneoplastic events occurring in the mucosa, and not as a result of regeneration.

The BBN-induced bladder carcinoma mouse model may be used to study other genes that are linked to the regulation of H19, e.g. Igf-2 (27,28) and p53 (31). It is a good model for human bladder cancer both by its similarity to the stages of cancer evolution in humans and the pathogenetic mechanism of chemical carcinogenesis. It may be further used as an animal model for trials of various modes of gene therapy against epithelial and stromal elements of the tumor.

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