Dietary Restriction Reduces Insulin-like Growth Factor I Levels, Which Modulates Apoptosis, Cell Proliferation, and Tumor Progression in p53-deficient Mice

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Abstract

Diet contributes to over one-third of cancer deaths in the Western world, yet the factors in the diet that influence cancer are not elucidated. A reduction in caloric intake dramatically slows cancer progression in rodents, and this may be a major contribution to dietary effects on cancer. Insulin-like growth factor I (IGF-I) is lowered during dietary restriction (DR) in both humans and rats. Because IGF-I modulates cell proliferation, apoptosis, and tumorigenesis, the mechanisms behind the protective effects of DR may depend on the reduction of this multifaceted growth factor. To test this hypothesis, IGF-I was restored during DR to ascertain if lowering of IGF-I was central to slowing bladder cancer progression during DR. Heterozygous p53-deficient mice received a bladder carcinogen, p-cresidine, to induce preneoplasia. After confirmation of bladder urothelial preneoplasia, the mice were divided into three groups: (a) ad libitum; (b) 20% DR; and (c) 20% DR plus IGF-I (IGF-I/DR). Serum IGF-I was lowered 24% by DR but was completely restored in the IGF-I/DR-treated mice using recombinant IGF-I administered via osmotic minipumps. Although tumor progression was decreased by DR, restoration of IGF-I serum levels in DR-treated mice increased the stage of the cancers. Furthermore, IGF-I modulated tumor progression independent of changes in body weight. Rates of apoptosis in the preneoplastic lesions were 10 times higher in DR-treated mice compared to those in IGF-I/DR- and ad libitum-treated mice. Administration of IGF-I to DR-treated mice also stimulated cell proliferation 6-fold in hyperplastic foci. In conclusion, DR lowered IGF-I levels, thereby favoring apoptosis over cell proliferation and ultimately slowing tumor progression. This is the first mechanistic study demonstrating that IGF-I supplementation abrogates the protective effect of DR on neoplastic progression.

Introduction

Diet contributes to approximately one-third of all cancer-related deaths worldwide (1). There are many components to the diet, but modulation of calories may be the most important (2). In rodents, DR can dramatically decrease tumor development in multiple models (3). Protection against tumor development by DR is a very general phenomenon, because spontaneously arising (4, 5) as well as chemically induced cancers (3) are suppressed by DR. Furthermore, DR protects against many types of cancers, ranging from mammary cancer to leukemia. It is noteworthy that even a modest reduction in caloric intake (20–40% DR) can have a significant impact on the development of cancer.

The mechanisms responsible for the anticancer properties associated with DR are unknown. Many physiological changes occur in response to DR, such as changes in pituitary-derived hormones and decreases in growth hormone, insulin, and IGF-I (6). Our laboratory is focusing on IGF-I, because we demonstrated that serum IGF-I levels are significantly reduced in DR-treated rats concomitant with a decrease in cancer incidence (7). IGF-I is known to inhibit apoptosis and also to stimulate proliferation (8). In addition, alterations in the IGF-I pathway are common in many cancers. For example, overexpression of the IGF-I receptor is found in human bladder cell lines (9). Both IGF-I and IGF-II signal through the IGF-I receptor, and IGF-II overexpression is associated with the development of multiple cancers of the pancreas (10), breast (11), and liver (12).

In this study, we addressed whether IGF-I reverses cancer prevention by DR. Transgenic mice heterozygous for the p53 gene were used, because they rapidly develop a high incidence of TCC of the bladder within 24 weeks after treatment with the bladder carcinogen p-cresidine (13). Prenecoplastic lesions were induced in mice after 16 weeks; then carcinogen treatment was stopped, and mice were fed either: (a) AL; (b) a modest 20% DR; or (c) a 20% DR with recombinant IGF-I administered via osmotic minipumps for 28 days. Fewer invasive TCCs were observed in the DR-treated mice compared to the IGF-I/DR-treated mice. Apoptosis was reduced 10-fold by IGF-I addition as compared to DR. Cell proliferation was also affected; there was 6-fold less proliferation in the DR-treated mice compared to the IGF-I/DR-treated mice. We conclude that DR decreases bladder cancer progression, partly due to changes in IGF-I. The mechanism of enhancing the cancer progression by IGF-I was through changes in the balance of cell proliferation and apoptosis.

Materials and Methods

p53 Transgenic Mice. TGS-p53border male mice were obtained from Taconic (Germantown, NY) at 7–10 weeks of age. For the first week, all mice were fed a diet containing 0.25% p-cresidine (Aldrich Chemical Co., Milwaukee, WI), followed by 15 weeks of 0.5% p-cresidine. An interim sacrifice was done at 15 weeks to determine the extent of preneoplastic changes. The objective was to stop exposure to p-cresidine after the induction of preneoplastic changes, but before cancers developed. Focal and atypical hyperplasia was observed at the 15-week time point; therefore, p-cresidine treatment was stopped, and the remaining mice were divided randomly into three groups: (a) AL; (b) 20% DR; and (c) 20% DR plus IGF-I, designated IGF-I/DR. IGF-I was administered via osmotic minipumps at a rate of 1 μg/h for 35 days [human recombinant IGF-I was kindly provided by Dr. Louis Underwood (University of North Carolina, Chapel Hill, NC)]. Mice were maintained in those treatment groups for 35 days after p-cresidine treatment cessation. There were 10 mice/treatment group.

All mice received a minipump (Alzet Corp, Palo Alto, CA) by surgical implantation; the AL and DR groups received saline in the minipump, whereas the IGF-I/DR-treated mice received human recombinant IGF-I. Animals were individually housed in polycarbonate cages and maintained in a pathogen-controlled environment. After acclimation for 3 days, the dietary treatments were imposed as described below. Deionized water was available AL. AL animals were allowed constant free access to unlimited feed, the consumption of which was estimated by daily weighing. The daily mean consumption of the AL group was multiplied by 0.80, and the resultant mass of food was offered to the diet-restricted animals at 7 am. daily for 35 days.

Two semipurified diets were formulated such that by feeding 0.8 g of the restricted diet (product D10010; Research Diets, Inc., New Brunswick, NJ) for...
every gram of AL diet (product D11359, Research Diets, Inc.), the diet-restricted animals would consume 20% fewer calories from carbohydrates and protein but equal amounts of fat, fiber, salts, and vitamins as did their AL counterparts. This mode of feeding was selected to exploit the ability of both protein but equal amounts of fat, fiber, salts, and vitamins as did their AL counterparts. This mode of feeding was selected to exploit the ability of both

The studies were conducted in a facility of the National Institute of Environmental Health Sciences accredited by the Association for Assurance and Accreditation of Laboratory Animal Care (Rockville, MD), with a protocol approved by the National Institute of Environmental Health Sciences Animal Care and Use Committee.

**Cell Proliferation, Tumor Staging, and Apoptosis.** Cell proliferation was assessed indirectly by BrdUrd incorporation using a previously reported method (15). BrdUrd-positive nuclei were quantified by microscopic analysis. The labeling index was determined by counting the number of positive nuclei of 500 cells. This was done by counting areas of hyperplasia. The regions of hyperplasia ranged from simple to diffuse and atypical. Hyperplastic and neoplastic nodules were evaluated separately. An average labeling index was established for each treatment group. There were 8–10 mice evaluated in each group. The tumors in the DR-treated mice were too small to count 500 cells, therefore the percentage of BrdUrd-positive cells was based on the number of cells in the tumor that were BrdUrd positive compared to the number that were negative. Care was taken to exclude inflammatory cells during the quantification of cell proliferation as inflammatory cells with the BrdUrd antibody. Tumor volume was determined by measuring the area of the carcinomas by using NIH image analysis software.

Apoptosis rates were determined by counting the number of apoptotic cells/500 cells in H&E-stained sections. Hyperplastic lesions were quantified and two other outside reviewers). In addition, apoptosis was scored based on nuclear morphology on the H&E slides. Cell proliferation was assessed by BrdUrd incorporation using a previously reported method (15). BrdUrd-positive nuclei were quantified by microscopic analysis. The labeling index was determined by counting the number of positive nuclei of 500 cells. This was done by counting areas of hyperplasia. The areas of hyperplasia ranged from simple to diffuse and atypical. Hyperplastic and neoplastic nodules were evaluated separately. An average labeling index was established for each treatment group. There were 8–10 mice evaluated in each group. The tumors in the DR-treated mice were too small to count 500 cells, therefore the percentage of BrdUrd-positive cells was based on the number of cells in the tumor that were BrdUrd positive compared to the number that were negative. Care was taken to exclude inflammatory cells during the quantification of cell proliferation as inflammatory cells with the BrdUrd antibody. Tumor volume was determined by measuring the area of the carcinomas by using NIH image analysis software.

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DR MODULATES TUMOR PROGRESSION IN p53-DEFICIENT MICE

Fig. 2. H&E-stained bladder tissues were used for pathology evaluations. a, normal urinary bladder showing transitional epithelium that was two to three cells thick (magnification, ×150). b, diffuse hyperplasia of the transitional epithelium lining the surface of the bladder. This was a typical change seen after 16 weeks of p-cresidine exposure (magnification, ×134). c, 35 days after p-cresidine cessation, focal marked hyperplasia and dysplasia of the transitional cell epithelium (magnification, ×135) were seen in an AL-fed mouse. d, early-stage (T1) carcinoma from an AL-fed mouse. Note the multiple nests of epithelial cells in the lamina propria (arrows) where the epithelium is forming a papilloma-like mass (magnification, ×67). e, stage T2 carcinoma invading the lamina propria (but not the serosa) in an IGF-1/DR-treated mouse (magnification, ×59). f, stage T3 carcinoma invading the lamina propria and muscularis in an IGF-1/DR-treated mouse (magnification, ×37).

Staging was based on the level of invasion. T1 was a TCC that was confined within the epithelial layer and did not invade the basement membrane. The T2-staged TCCs were defined as those that had invaded the lamina propria, and a T3 tumor was one that had invaded past the lamina propria into the muscularis. Criteria for tumor staging were taken from International Union Against Cancer description of human bladder tumors (16).

Serum IGF-I. Serum was collected at the time of necropsy and frozen at −80°C until analysis. Quantification of serum IGF-I was achieved using a RIA specifically designed for IGF-I (Nichols Institute Diagnostics, Capistrano, CA).

Fied for apoptosis. There were no apoptotic cells in the two TCCs from the DR-treated group; therefore, comparisons for apoptosis from TCCs could not be made between groups. Cells were considered apoptotic if they possessed one or more of the following characteristics: (a) condensed nuclei; (b) crescent appearance of chromatin; (c) emargination of chromatin along nuclear membrane and nuclear fragmentation; and (d) presence of apoptotic bodies or phagocytosis by neighboring cells. We elected to identify apoptosis based on changes in nuclear morphology because we have found this to be the most stringent method for identifying apoptosis in formalin-fixed tissues.
Statistics. Statistical analysis was performed independently by Analytical Sciences, Inc. Wilcoxon’s rank-sum test or Student’s t test was used to evaluate statistical significance.

Results

The experimental design for the study was created to capture events that modulate tumor progression from preneoplasia to malignant cancers (Fig. 1a). The mice on an AL diet were treated with the bladder carcinogen p-cresidine for 15 weeks, which was determined in a pilot experiment to induce a high incidence of hyperplasia/dysplasia in the bladder. At this point, carcinogen treatment was stopped, and the mice were randomly divided into three groups: (a) AL; (b) 20% DR; and (c) IGF-1/DR. Body weights were monitored throughout the study. Mice fed AL steadily increased in weight: initial average weight was 28.6 ± 0.33 g/mouse; the final average weight at the end of the study was 38 ± 1.31 g/mouse. With a modest DR of 20%, the animals neither gained nor lost weight. The body weight of DR-treated mice at the end of the study was 30 ± 0.53 g/mouse, which was significantly less than that of AL-fed mice (38 ± 1.31 g/mouse; P < 0.05). The body weights of DR- and IGF-1/DR-treated mice were similar, indicating that IGF-1 supplementation to DR-treated mice was insufficient for weight gain. The average body weight at the end of the study for the IGF-1/DR-treated mice was 31.93 ± 0.36 g/mouse.

We have previously reported that a 40% DR in rats results in approximately a 50% reduction in serum IGF-1 (5). In this study, we chose a more modest DR of 20% that reduced serum IGF-1 by 26% in the mice. Serum IGF-1 was lowered from 367 ± 31 ng/ml in the AL-fed mice to 271 ± 34 ng/ml in DR-treated mice (P < 0.05). IGF-1 in the IGF-1/DR-treated (390 ± 25 ng/ml) mice was restored to AL levels using osmotic minipumps (Fig. 1b). Thus, serum IGF-1 levels were essentially the same in the AL-fed mice versus the IGF-1/DR-treated mice.

In a previous study, we showed that the p53 heterozygous mice do not develop spontaneous bladder cancers, whereas treatment of AL-fed mice with p-cresidine resulted in a 90–100% incidence of TCC (13). In this study, we observed that normal bladder urothelia is typically two to three cell layers thick (Fig. 2a). After 16 weeks of exposure to p-cresidine, the earliest change was simple, diffuse hyperplasia consisting of a uniform thickening of the surface epithelial cell layer (Fig. 2b). Twenty-eight days after p-cresidine cessation, focal hyperplasia occurred in addition to localized dysplasia (Fig. 2c). TCCs were also evident. The criteria for TCC were based on nuclear atypia, abnormal cellular morphology, and invasiveness. TCCs were also staged based on the degree of tumor invasion. Invasion into but not beyond the urothelial layer was considered T1 (Fig. 2d), whereas invasion into the lamina propria was considered T2 (Fig. 2e). More extensive invasion through the lamina propria and into the muscularis was considered T3 (Fig. 2f).

TCCs were evaluated for stage, multiplicity, incidence, and size. The incidence of tumor in the AL group was 0 of 10 T1, 4 of 10 T2, and 1 of 10 T3 TCCs (Table 1). In contrast, DR-treated mice TCCs were much less invasive. There were 2 of 10 T1, and no T2, or T3 TCCs. IGF-1/DR-treated mice had markedly more invasive TCCs compared to DR-treated mice; there were 0 of 10, 7 of 10, and 2 of 10 T1, T2, and T3 lesions. The stage of TCC was statistically different between DR- and IGF-1/DR-treated mice as well as between DR-treated and AL-fed mice (P < 0.05). The trend was for DR-treated mice to have a lower-stage TCC than AL-fed or IGF-1/DR-treated mice. Because some mice had more than one tumor, SCC staging was performed on individual tumors. Tumor multiplicity was evaluated next. Multiple tumors were observed in the IGF-1/DR (3 of 10) and AL (1 of 10) groups, but not in the DR-treated mice. The tumor incidence was 2 of 10, 6 of 10, and 4 of 10 in the DR-treated, IGF-1/DR-treated, and AL-fed mice, respectively. The tumor incidence, although not statistically different, was suggestive of an IGF-1-related increase compared to DR. IGF-1 also influenced the size of the TCCs. The median size TCC was 0.919 mm² in the IGF-1/DR-treated mice, compared with 0.214 mm² in the DR-treated mice (Table 1). Although there was a greater than 4-fold difference in the median size of TCC in the IGF-1/DR treatment group compared to that of the DR-treated group, statistical significance was not reached (P > 0.07) due to considerable intra-animal variability. Taken together, the weight of evidence presented supports the hypothesis that IGF-1 restoration impacted tumor progression when stage, incidence, and size are considered collectively.

The changes observed in TCC size and stage may reflect an imbalance in the rates of cell proliferation and apoptosis. Apoptosis was measured in the histological specimens and in some areas of focal hyperplasia; apoptosis in the DR-treated mice was quite profound (Fig. 3a, inset). DR increased apoptosis 10-fold compared to that of the AL control (7.355 ± 1.3 versus 0.694 ± 0.08; P < 0.005; Fig. 3a). In contrast, IGF-1/DR reduced the rate of apoptosis 10-fold relative to that of DR (0.834 ± 0.149 versus 7.355 ± 1.3; P < 0.005; Fig. 3a). There were no differences in apoptosis rates between the AL-fed and IGF-1/DR-treated groups. Apoptotic cells were most prevalent in areas of focal hyperplasia and in TCC. It is noteworthy that apoptosis was not often observed in the normal bladder epithelial cells.

Cell proliferation was evaluated by incorporation of BrdUrd. BrdUrd labeling is an indirect measurement of proliferation based on the incorporation of BrdUrd (a thymidine analogue) during DNA synthesis. IGF-1 restoration resulted in a 6-fold increase in cell proliferation compared to DR (Fig. 3b) 312 ± 79 versus 46 ± 40, yielding a proliferation index of 62 versus 59% (P < 0.001), respectively. High rates of BrdUrd-labeled cells were observed in hyperplastic foci taken from IGF-1/DR-treated mice (Fig. 3b, inset). Labeling rates were the same (62 versus 59%) in IGF-1/DR-treated groups and in AL-fed mice (312 ± 79) and in AL-fed mice (297 ± 110); hence, IGF-1 alone completely restored cell proliferation rates. TCCs were also analyzed for cell proliferation, but there was considerable variability within and between treatment groups. This may be due to differences in TCC growth, vascular supply, or BrdUrd metabolism within the tumors. Variations in...
There were 8–10 mice evaluated per treatment group.

There was no difference in cell proliferation between IGF-I/DR-treated and AL-fed mice. A higher proliferation index in hyperplastic foci was observed in IGF-I/DR-treated mice (62%) and AL-fed mice as compared to the IGF-I/DR-treated and AL-fed mice (P < 0.001, Student’s t test).

Mouse (magnification, X250). Cell proliferation was significantly lower in the DR-treated mouse (59%) compared to DR-treated mice (9%). In areas of hyperplasia, BrdUrd staining was often intense. Inset, an example of proliferation activity from an IGF-I/DR-treated mouse (magnification, X250). Rates of apoptosis were higher in preneoplastic and neoplastic cells compared to normal urothelial cells. High rates of apoptosis in preneoplastic cells relative to normal cells were previously reported in our laboratory (19) and by others (10). In addition, we found that apoptosis and cell proliferation rates both increased with neoplastic progression. The apoptotic index of human TCC also increases with increasing stage (20).

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The role of the IGF-I receptor and its ligands IGF-I and IGF-II in modulating apoptosis has been demonstrated in a variety of experimental models. Apoptosis is decreased in cell lines that overexpress the IGF-I receptor (21). Furthermore, apoptosis is induced by the use of antisense oligonucleotides to the IGF-I receptor (22) and by expression of a dominant negative mutated IGF-I receptor (23). Studies with the IGF-I and IGF-II ligands have been somewhat more limited. IGF-I protects against c-myc-induced (24) and etoposide-induced apoptosis in mouse fibroblasts (25). Use of antisense oligonucleotides to IGF-II mRNA reduces pancreatic tumor cell proliferation and enhances apoptosis in vivo (10). Recently, IGF-I was shown to inhibit apoptosis during mammary gland involution in transgenic mice (26). Hence, manipulation of the IGF-I receptor and its ligands results in the suppression of apoptosis.

Growth factors such as fibroblast growth factor (27), epidermal growth factor (28), and vascular endothelial growth factor (29) are associated with bladder cancer. Both proliferation and apoptosis rates are modulated by growth factors, but more recently, evidence is accumulating for the role of growth factors in tumor invasion and metastasis. Acidic fibroblast growth factor stimulates invasion of the bladder cancer cell line T24 into primary urothelia cultures (30). Both IGF-I and IGF-II are chemotactic for a wide variety of cancers and endothelial cells in vitro (31), but the association of IGFs in bladder cancer invasion has not been investigated. Overexpression of the IGF-I receptor (9) and IGF-II (32) has been reported in the J82 bladder cancer cell line. This is interesting, because the J82 cell line was isolated from a high-grade, invasive, primary TCC, which is consistent with the hypothesis that IGF-I signaling influences the severity of bladder cancer. In this study, IGF-I increased the TCC staging based on the criteria of tumor invasion; this is particularly important because tumor invasion carries a poor prognosis in humans (33). Collectively, these data suggest that IGF-I contributes to the progression of bladder cancer and broadens the role of IGF-I in carcinogenesis, because it has been previously associated with other cancers such as breast, colon, liver, and lung carcinomas (34).

Because IGF-I influences bladder cancer development in transgenic

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ratio of apoptotic cells/total cells counted in hyperplasia</th>
<th>Average % BrdUrd-positive nuclei/500 cells counted/mouse</th>
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<tbody>
<tr>
<td>AL</td>
<td>4.50 ± 0.12</td>
<td>22</td>
</tr>
<tr>
<td>DR</td>
<td>7.50 ± 0.22</td>
<td>62</td>
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<tr>
<td>IGF-I/DR</td>
<td>8.75 ± 0.27</td>
<td>62</td>
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BrdUrd labeling also suggest that the tumors had developed at least 3 days before BrdUrd pulse labeling.

**Discussion**

Diet has a potent influence on the development of cancer, possibly through the elimination of preneoplastic and neoplastic cells. In rats treated with a liver carcinogen, DR alters the balance of tumor growth by decreasing cell proliferation and stimulating apoptosis (17), resulting in the elimination of preneoplastic foci (18). In this study, we found that DR increased the rates of apoptosis 10-fold. Because DR also decreased IGF-I serum levels in this and a previous study (7), we tested whether restoration of IGF-I by an osmotic minipump could affect the apoptosis rate of bladders possessing preneoplastic changes. We found that an administration of IGF-I dramatically suppressed the high rates of apoptosis observed in DR-treated mice. It is noteworthy that we did not observe a complete remodeling of the bladder epithelial cells by removal of preneoplastic cells, as was observed in the liver (18), possibly because the liver has a greater capacity to regenerate than the bladder.

The rates of apoptosis were higher in preneoplastic and neoplastic cells compared to normal urothelial cells. High rates of apoptosis in preneoplastic cells relative to normal cells were previously reported by our laboratory (19) and by others (10). In addition, we found that apoptosis and cell proliferation rates both increased with neoplastic progression. The apoptotic index of human TCC also increases with increasing stage (20).

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Because IGF-I influences bladder cancer development in transgenic
mice, approaches to lower IGF-I may be of benefit in humans. Approaches could include lowering serum IGF-I levels or inhibiting the IGF-I receptor. Drugs that reduce serum IGF-I levels in humans such as tamoxifen (35) and octreotide (36) may be of benefit. Tamoxifen enhances the cytotoxicity of classic chemotherapeutic drugs used to treat bladder cancer in vitro (37). We recently reported that IGF-I modulates apoptosis in human breast cells (38) induced by tamoxifen, methotrexate, 5-fluorouracil, or camptothecin. Because chemotherapeutic drugs kill cancer cells by the induction of apoptosis, it follows that the lowering of antiapoptotic peptides, such as IGF-I, would sensitize preneoplastic and neoplastic cells to apoptosis. The bladder is particularly amenable to such experimental treatments, because localized therapy is possible by intravesical administration, thus minimizing side effects. Certainly the least invasive of all strategies is through dietary modifications, which may prove very important in protecting against the progression and recurrence of bladder cancer. DR has many beneficial effects, which include an extension of life span and protection against a variety of cancers (3), including bladder cancer, as we have now shown.

In conclusion, a major factor in DR modulation of cancer is the lowering of IGF-I. The mechanisms of IGF-I in tumor progression depend on both a stimulation of cell proliferation and an inhibition of apoptosis that result in larger, more invasive TCCs. These data implicate IGF-I as an important controller of bladder cancer; therefore, disruption of IGF-I signaling may slow tumor progression and improve therapy.

Acknowledgments

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References

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