Tumor suppression and apoptosis of human prostate carcinoma mediated by a genetic locus within human chromosome 10pter–q11

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Communicated by Frank H. Ruddle, Yale University, New Haven, CT, November 13, 1995

ABSTRACT Prostate cancer is the second leading cause of male cancer deaths in the United States. Yet, despite a large international effort, little is known about the molecular mechanisms that underlie this devastating disease. Prostate secretory epithelial cells and androgen-dependent prostate carcinomas undergo apoptosis in response to androgen deprivation and, furthermore, most prostate carcinomas become androgen independent and refractory to further therapeutic manipulations during disease progression. Definition of the genetic events that trigger apoptosis in the prostate could provide important insights into critical pathways in normal development as well as elucidate the perturbations of those key pathways in neoplastic transformation. We report the functional definition of a novel genetic locus within human chromosome 10pter–q11 that mediates both in vivo tumor suppression and in vitro apoptosis of prostatic adenocarcinoma cells. A defined fragment of human chromosome 10 was transferred via microcell fusion into a prostate adenocarcinoma cell line. Microcell hybrids containing only the region 10pter–q11 were suppressed for tumorigenicity following injection of microcell hybrids into nude mice. Furthermore, the complemented hybrids underwent programmed cell death in vitro via a mechanism that does not require nuclear localization of p53. These data functionally define a novel genetic locus, designated PAC1, for prostate adenocarcinoma 1, involved in tumor suppression of human prostate carcinoma and furthermore strongly suggest that the cell death pathway can be functionally restored in prostatic adenocarcinoma.

The initiation and progression of human cancer is a multistep process that must involve genetic alterations of critical genes controlling the destiny of defined cell lineages with regard to cellular proliferation, differentiation, or death. Elucidation of the genetic mechanisms underlying these growth control processes is crucial to understanding the origins of the malignant state. Prostate cancer is the most common cancer in men and, with rising incidence, is the second leading cause of male cancer deaths in the United States (1, 2). The search for specific genetic changes associated with prostate cancer has failed to uncover a single high-frequency event (with the exception of overproduction of prostate-specific antigen).

Cytogenetic and loss of heterozygosity studies, however, have pointed to several regions of the human genome that could contain tumor suppressor genes involved in the etiology of prostate cancer (3–8). Alleotyping studies such as that of Kunimi et al. (4) in prostate adenocarcinoma indicate candidate regions to include chromosome 8p(50–65%), 10p(55%), 10q(30%), 16q(55–60%), and 18q(43%) (4). High-frequency loss of heterozygosity and homozygous deletion have been observed within chromosome 8p22 at the MSR locus in a prostate tumor (9). Recently, a candidate tumor suppressor gene MXII was identified within 10q24 (10). Mutation of the MXII gene, a negative regulator of the MYC oncoprotein, was found in 4 of 10 primary prostate tumors examined (10).

Cytogenetic analyses of human prostatic adenocarcinoma have also implicated nonclonal aberrations of several different human chromosomes. A recent study by Lundgren et al. (11) indicated consistent aberrations of chromosomes 1, 7, 8, and 10. Breakpoints were identified at 7q22 and 10q24. The cumulative findings of cytogenetic analysis and allele loss studies suggest a consistent association with genetic loci on a subset of human chromosomes, the most consistent correlations being with chromosomes 8 and 10 and prostate carcinoma.

For our experiments, a functional genetic approach was taken to identify tumor suppressor loci involved in prostate cancer. Previous studies have shown that it is possible to complement the genetic defect in particular human cancers, which show high-frequency allele loss on a defined chromosome, by the microcell fusion (12, 13) of a normal copy of that chromosome containing a putative tumor suppressor gene (14–21). We report that the introduction of human chromosome 10 or a subchromosomal fragment of 10 (encompassing 10pter–q11) into a prostate adenocarcinoma cell line resulted in a dramatic suppression of tumor formation following injection of hybrid clones into nude mice. Furthermore, we provide evidence that complemented hybrid clones underwent programmed cell death in vitro via a mechanism of apoptosis that does not require nuclear localization of p53. Induction of phagocytosis of apoptotic cells by neighboring prostate cells was observed in vitro, a process reminiscent of prostatic involution in response to androgen withdrawal (22, 23).

MATERIALS AND METHODS

Cell Lines and Construction of Hybrids. The prostatic adenocarcinoma cell line PC-3 was established from the poorly differentiated adenocarcinoma from a vertebral body metastasis in a patient with hormone-insensitive prostate cancer (24). The PC-3 cell line, obtained from the American Tissue Culture Collection, clearly contained two subpopulations of cells: one population containing a modal number of 60 and a second tetraploid population. PC-3 contained no recognizable chromosome 10 by G-banding analysis. A subcloned line (PC-3H) was isolated for these studies that contained a modal

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number of 60 chromosomes and served as recipient for the transfer of human chromosome 10 or a defined region of chromosome 10 via microcell fusion.

The following microcell hybrids were used as donor lines for microcell transfer into PC-3H: HA(10)A, which contains an intact neo-marked human chromosome 10 in the A9 mouse cell background; HA(10p)A, which contains the region 10pter-q11 in the mouse A9 cell background. The centric fragment is dominantly marked with neo, which is integrated near the centromere on the long arm of chromosome 10; HA(8)A, a monochromosomal hybrid containing a neo-marked human chromosome 8 in the A9 cell background.

**Microcell-Mediated Chromosome Transfer.** Conditions for efficient micronucleation of A9-based hybrids and subsequent enucleation have been previously outlined (for review, see ref. 25). Fusion of HA(10)A or HA(10p)A microcells to recipient PC-3H cells was accomplished by a suspension microcell fusion technique (13, 25).

**Characterization of Microcell Hybrid Clones.** For chromosome painting, human chromosome 10-specific DNA from HA(10)A was amplified by PCR using inter-Alu primers (26). Hybridization and detection was carried out as described (21).

**Tumorigenicity Assays.** The PC-3H line and PC(10) series hybrids were injected at 5 × 10⁶ cells into each of three 6- to 8-week-old athymic nude mice (Harlan). Injections were performed subcutaneously and tumor volumes were monitored biweekly using calipers as described (21).

**Apoptosis Assays.** The DNA ladder protocol was that of Fernandez et al. (27). Detection of DNA fragmentation in situ followed the procedure of Gavrieli et al. (28).

**p53 Immunostaining and Western Blot Analysis.** Immunostaining of p53 in PC-3H and PC(10) hybrids was accomplished using a mouse monoclonal antibody against human p53 (DO1) (Santa Cruz Biotechnology). The bound antibody was detected with an anti-mouse antibody conjugated to horseradish peroxidase, according to the manufacturer's directions. BCL-2 protein levels were detected in parental and microcell hybrid clones by Western blot analysis as described by Hockenberry et al. (29).

**RESULTS**

**Construction and Characterization of Monochromosomal Microcell Hybrid Clones.** A subcloned line of the prostatic adenocarcinoma PC-3 was chosen as recipient for microcell fusion of human chromosome 10. PC-3H contained no intact copies of human chromosome 10; however, small fragments of chromosome 10 interspersed throughout the genome were apparent by chromosome painting using chromosome 10-specific DNA as a probe (Fig. 1A).

HA(10)A was used as a donor cell line for microcell fusion into PC-3H [PC(10) series hybrids]. Twenty hybrid clones isolated from a single microcell fusion experiment were examined by high-resolution cytogenetics for the presence of an intact chromosome 10. Twelve of 20 clones contained an intact human chromosome 10 (Fig. 1B).

**Introduction of Human Chromosome 10 Results in Prostate Carcinoma Tumor Suppression.** To test the effect of the introduced chromosome on the tumorigenic potential of the PC-3H cells, three PC(10) hybrids and parental PC-3H cells were injected subcutaneously in triplicate (at 5 × 10⁶ cells per animal) into 6- to 8-week-old athymic nude mice. Tumor volumes were followed weekly and the tumors were excised 8 weeks postinjection and established in culture. The first of three separate nude mice experiments is illustrated in Table 1. Tumor volumes and tumor wet weights were calculated from an average of three animals injected per cell line tested. In the first series of injections, the parental subcloned line PC-3H was injected subcutaneously into each of six animals with an average tumor wet weight at the end of 65 days equal to 0.61 g.

In total, PC-3H was injected into 12 mice. Only 1 of the 12 mice injected failed to form any tumor. Eleven of the 12 mice injected consistently formed large tumors with average tumor wet weights in the range of 0.61–1.3 g (56–65 days postinjection). Injection of monochromosomal hybrids containing an intact chromosome 10 into athymic nude mice, however, showed dramatic tumor sup-
pression (Fig. 2 and Table 1). Two of three PC(10) hybrids (PC(10)2 and PC(10)7) showed >10-fold suppression of tumor formation relative to parental PC-3H cells. The average tumor wet weights of three animals injected per cell line were 0.06 and 0.05 g, respectively (Table 1). The remaining PC(10)1 hybrid formed tumors of intermediate size between PC-3H and the suppressed PC(10) clones. PC(10)1 contained an intact chromosome 10 at the time of injection. However, an increase in chromosome instability was clearly evident in this clonal population. Fourteen percent of metaphases examined in PC(10)1 showed evidence of chromosome breakage compared with 4% in PC(10)2 and 2% in PC(10)7. Thus, an increased chromosome instability, leading to a more rapid loss of the introduced chromosome from the cells in vivo, could potentially explain the intermediate tumor suppression observed in PC(10)1. Cytogenetic analysis performed on tumors from hybrid clones explanted on day 65 indicated loss of the introduced chromosome 10 in 100% of metaphases examined in each of three PC(10) clones (data not shown). In fact, in one experiment, tumor volumes for PC(10)7 increased from 54.1 mm³ at day 51 to 228 mm³ at day 58, indicating a more rapid expansion of the hybrid population in vivo at the end of the experiment concomitant with loss of the introduced chromosome. Thus, introduction of chromosome 10 into PC-3H resulted in a suppression of the malignant phenotype in vivo; furthermore, loss of the introduced chromosome was found in all hybrid metaphases derived from the explanted tumors, indicating that tumor suppression was dependent on retention of the normal copy of chromosome 10. These data define a functional tumor suppressor locus within chromosome 10 involved in the etiology of human prostate cancer.

**Fragment-Containing Microcell Hybrids Used to Limit the Region Within Chromosome 10 Containing a Functional Tumor Suppressor Gene.** To regionally localize the tumor suppressor gene within chromosome 10, microcell hybrid clones were first constructed in the A9 cell background carrying a deletion of the introduced chromosome 10. The HA(10)p series hybrids were first rescreened to detect any clones that might contain a deletion of the introduced chromosome in the A9 cell background. One hybrid clone, HA(10)pA, was obtained that contains a large terminal deletion of most of the long arm of chromosome 10 and retains only the region 10pter–q11 by high-resolution cytogenetics as well as by chromosome painting using HA(10)pA as a probe onto normal human metaphases (Fig. 1C). HA(10)pA was then used as a donor cell line to transfer this small marked centric fragment of chromosome 10 into PC-3H (PC(10)p series hybrids).

Two PC(10)p hybrid clones containing the introduced region 10pter–q11 in the PC-3H background were each injected into three athymic nude mice along with PC(10)7 and PC-3H cells. As illustrated in Fig. 2 and Table 1, the introduction of the region 10pter–q11 mediated tumor suppression equivalent to that observed with the introduction of the intact chromosome 10. This experiment was repeated using PC(10)pC and PC(10)pD for a total of six mice injected and PC(10)7 for a total of nine mice injected. Consistent tumor suppression was observed following introduction of chromosome 10 and chromosome 10pter–q11. These results regionally define a genetic locus within human chromosome 10 involved in the etiology of prostate cancer. We have designated this genetic locus PAC1, for prostatic adenocarcinoma 1.

**A Genetic Locus Within Chromosome 10pter–q11 Restores the Programmed Cell Death Pathway in Prostate Carcinoma Cells.** In addition to the functional studies in vivo, we further examined the PC(10) and PC(10)p series hybrids for growth in vitro in an attempt to provide insight into the mechanism of action of PAC1. The first clues to the phenotypic changes in PC-3H growth in vitro were evident upon clonal expansion of PC(10) hybrids. Nine of 17 PC(10) clones died following expansion from six-well plates at one to three absolute passages in vitro. We hypothesized that both in vitro cell death and in vivo tumor suppression could be explained if the genetic complementation in the PC(10) series clones was the result of activation of a cell death program. In this regard, prostatic secretory epithelial cells and androgen-dependent prostate carcinomas undergo apoptosis in response to androgen deprivation (22, 23). Furthermore, during disease progression most prostate carcinomas become androgen independent and refractory to further therapeutic manipulations (30). To test whether genes on chromosome 10 could restore the cell death pathway in PC-3H, three PC(10) clones and two fragment-containing clones PC(10pter–q11) clones were expanded for in vitro apoptosis assays. As a control, a hybrid was also constructed containing an intact neo-marked human chromosome 8 in the PC-3H background [PC(8)B]. DNAs isolated from these hybrids and PC-3H were analyzed by agarose gel electrophoresis for the presence of the characteristic DNA ladder indicative of apoptosis. All hybrids containing chromosome 10 as well as all fragment-containing hybrids displayed the typical endonuclease-induced DNA fragmentation often seen in cells undergoing apoptosis (30, 31). DNA ladders were observed in all hybrids even in 10% serum, whereas no laddering was observed in PC-3H or PC(8)B using the same conditions (Fig. 3).

Hybrid clones were also analyzed by the TUNEL technique to visualize DNA fragmentation in situ. Significantly, all PC(10) and PC(10)p hybrids examined showed positive staining that indicated not only the presence of fragmented chromatin but also of apoptotic bodies (Fig. 4A and B). TUNEL staining detected fragmented chromatin and apoptotic bodies in the cytoplasm of hybrid cells with intact nuclei, suggesting that cells undergoing apoptosis were being phagocytosed by neighboring cells with intact nuclei (Fig. 4A and B). Phagocytosis of apoptotic cells has been commonly observed in vivo.
apoptosis in cell carcinoma mediated tumor strongly functionally define 32). 31, studies and PC(lOp) and the developing numbered lanes). serum PC(lOp)D (lanes 1 (lanes 1 and 2). (B) PC(10)7 (lanes 1 and 2), PC(10)pD (lanes 3 and 4), and PC(10)pC (lanes 5 and 6). (C) PC(8)B (lanes 1 and 2). Growth conditions were in either 10% fetal bovine serum (odd-numbered lanes) or 0.1% fetal bovine serum (even-numbered lanes). Lanes M, size markers.

and it is thought to be an efficient clearing mechanism to rid the developing tissue of large numbers of dying cells (22, 23, 31, 32). Electron microscopic analysis confirmed TUNEL studies and indicated apoptosis and phagocytosis in PC(10) and PC(10)p series hybrids (data not shown). These data functionally define a genetic locus within human chromosome 10pter-q11 that directs the programmed cell death of prostatic carcinoma cell line PC-3H. Furthermore, results from these studies strongly suggest that one mechanism of PACI-mediated tumor suppression could be by induction of apopto-

sis. Finally, by analyses of these defined microcell hybrids, we have shown that the cell death pathway can be functionally restored in prostatic adenocarcinoma.

Apoptosis in PC(10) Series Hybrids Is Independent of Nuclear Localization of p53 or Downregulation of BCL2. The protein product of the tumor suppressor gene p53 has been shown to have a major role in regulation of apoptosis in other systems (33). Alterations in the p53 gene and aberrant expression of p53 protein have been associated with poorly differentiated, metastatic, androgen-independent prostatic tumors (34–37). For these reasons, hybrid clones were examined for changes in levels of p53 protein coincident with the introduction of the region 10pter–q11 into PC-3H cells. Immunohistochemistry was carried out to detect p53 protein in the PC-3H and PC(10) hybrid clones. Cells were cultured in vitro using conditions similar to those used to detect DNA fragmentation and then incubated with a monoclonal antibody against p53 (DO1) and a secondary antibody conjugated to horseradish peroxidase. No detectable p53 protein was observed in PC-3H cells (Fig. 5A) when cells were grown in 10% or 0.1% fetal bovine serum. However, clearly detectable p53 protein was observed in hybrids containing either the intact human chromosome 10 or the 10pter–q11 region (Fig. 5 C and D). These data suggest that either the transcription of p53 is upregulated or the p53 protein is stabilized in response to introduction of a genetic locus within chromosome 10. p53 protein, however, fails to localize to the nucleus in hybrid cells.

In addition to its role in induction of apoptosis, wild-type p53 seems to be required for induction of G1 arrest in the mammalian cell cycle. Rapid induction of stable p53 protein following UV irradiation of cells has been documented and suggests that the p53 pathway is required as a checkpoint in G1 to prevent entry into S phase until UV-damaged DNA can be repaired (38). To determine whether p53 in PC-3H cells is capable of nuclear localization, UV was used to induce up-

Fig. 3. Induction of apoptosis in PC(10) and PC(10)p microcell hybrids. (A) PC-3H (lanes 1 and 2). (B) PC(10)7 (lanes 1 and 2), PC(10)pD (lanes 3 and 4), and PC(10)pC (lanes 5 and 6). (C) PC(8)B (lanes 1 and 2). Growth conditions were in either 10% fetal bovine serum (odd-numbered lanes) or 0.1% fetal bovine serum (even-numbered lanes). Lanes M, size markers.

Fig. 4. Detection of DNA fragmentation in situ and phagocytosis in prostate hybrid cells. (A and B) Presence of apoptotic bodies and phagocytosis in PC(10) series hybrids.
regulation of p53 in PC-3H cells. PC-3H cells were irradiated with UV radiation (50 J/m²). As a control, LNCaP cells, another prostatic adenocarcinoma line, and early passage human fibroblasts were also irradiated under the same conditions. Six hours post-UV irradiation, p53 protein was detected in the nuclei of primary human fibroblasts and LNCaP cells as expected for wild-type p53 (data not shown); however, in PC-3H cells, p53 protein was highly expressed only in the cytoplasm (Fig. 5B). Thus, p53 protein in PC-3H cells is upregulated by introduction of chromosome 10, chromosome 10pter–q11, and presumably PAC1; however, the p53 protein localizes to the cytoplasm.

Apoptotic cell death in PC(10) and PC(10p) hybrids could also be the result of PAC1-directed downregulation of BCL2, a protooncogene that has been shown to rescue certain cell types from apoptotic death (34, 35). Previous work has shown that BCL2 is not expressed in normal prostate secretory epithelial cells. However, BCL2 is highly expressed in relapsed, androgen-independent prostate cancers (30). To address the involvement of BCL2 in this system, levels of BCL2 protein in PC-3H and PC(10) hybrids were studied by Western blot analysis (data not shown). Results indicated that BCL2 protein is expressed at basal levels in PC-3H and hybrid cells. These data indicate that PC-3H cells are not refractory to apoptosis due to upregulation of BCL2 and that the induction of a cell death pathway in PC(10) and PC(10p) hybrids does not involve downregulation of BCL2.

DISCUSSION

Our studies indicate the involvement of the genetic locus PAC1 in the tumor suppression and induction of apoptosis in the prostate adenocarcinoma cell line PC-3H. This study provides functional evidence for a tumor suppressor gene within human chromosome 10 involved in prostate cancer. We furthermore show that the pathway to programmed cell death can be functionally restored in PC-3H and implicate a novel mechanism that does not require nuclear localization of p53. A previous report documented that PC-3 contains an exon 5 mutation in p53 that would cause a frameshift in the amino acid sequence (39). Sequence analysis in our laboratory has confirmed the exon 5 mutation in codon 138 in the subcloned line PC-3H. The frameshift mutation generates a premature stop codon in exon 5, well before the nuclear localization signal in exon 10 (A.M.K., unpublished results). The consequence of this mutation would then be a truncated p53 protein that localizes to the cytoplasm. We furthermore report upregulation of p53 protein in PC-3H in response to UV and following the introduction of PAC1, although the p53 protein remains in the cytoplasm. One possible explanation for these results could be that p53 has a nontranscriptional activation-dependent role in apoptosis as proposed by Caelles and coworkers (40), perhaps in a cytoplasmic signaling pathway. An alternative explanation is that the tumor suppression and apoptosis in PC-3H cells does not require wild-type p53. In this regard, previous work has shown that the programmed cell death of murine androgen-dependent prostatic glandular cells in vivo following androgen ablation does not require wild-type p53 (41). However, the apoptosis observed in these in vivo studies was the result of growth factor withdrawal; the cell death phenotype in the PC(10) and PC(10p) hybrids does not require an inducing agent. These results, then, taken together would suggest that the tumor suppressor locus within 10pter–q11 mediates its effect on apoptosis either independently or upstream of p53. Supportive of these data is the finding that p53 mutations in prostate cancer have been found in the later stages of disease (35–36).

Definition of PAC1 was made possible by the use of genetic complementation via microcell fusion experiments. Although allele loss studies and cytogenetic analysis can...
implicate specific chromosomal regions as sites for putative tumor suppressor loci, these studies point to multiple regions on different chromosomes that are potentially involved in prostate cancer. Microcell fusion of defined chromosomal regions can functionally define the region containing tumor suppressor genes important in the genesis of this cancer. In addition, these studies and others indicate that complemented microcell hybrid clones are valuable to gain insight into the phenotypic changes in vitro and in vivo that accompany the introduction of a single copy of the normal genetic locus.

Defined deletion microcell hybrids can be very useful as starting materials for the physical mapping of PAC/ and to determine whether subsequent deletions in PC hybrids allow separation of the phenotypes of tumor suppression and apoptosis or indicate single gene involvement. Such deletion hybrids should be extremely valuable in combination with functional assays to isolate candidate tumor suppressor genes involved in prostate cancer.

A.M.K. is supported by grants from the National Institutes of Health and The University of Texas M. D. Anderson Physicians Referral Service. Y.S. was supported by National Institutes of Health Postdoctoral Fellowship CA09299. T.J.M. is a Pew Scholar in the Biomedical Sciences and is supported by CAPCURE, The Association for the Cure of Cancer of the Prostate. M.C.M. is supported by National Institutes of Health Predoctoral Fellowship CA09255.