Tumorigenicity and Oncogene Expression in Pediatric Cancers

Steven R. Pasquale, Gary R. Jones, Claus-Jens Doersen, and Bernard E. Weissman

Division of Hematology-Oncology, Children's Hospital of Los Angeles and Department of Microbiology, USC School of Medicine [S. R. P., G. R. J., B. E. W.], Los Angeles, California 90033, and Corporate Research Division, Procter and Gamble Company, Miami Valley Laboratories, Cincinnati, Ohio 45239-8707 [C-J. D.]

ABSTRACT

Cytogenetic and epidemiological studies of pediatric cancers have implicated a loss of genetic information in the development of these tumors. In contrast, other studies have shown that activation of endogenous oncogenes is a common event in these cancer cells. The technique of somatic cell hybridization provides a model for investigating the interaction between loss of genetic elements and oncogene activation in pediatric cancers. A variety of human-human cell hybrids were formed between a tumorigenic adult carcinoma and representative tumorigenic pediatric cell lines. All hybrid cells were completely suppressed for tumor-forming ability when assayed in nu/nu (nude) mice. When the expression of the N-myc, c-myc, and sis oncogenes and tumorigenicity were examined in the same hybrid lines, no correlation was found, suggesting that the expression of these oncogenes in these hybrid cells did not appear to be controlled by putative “tumor suppressor” genes. Thus, tumorigenicity behaves as a recessive genetic trait in pediatric cancers. Furthermore, different genetic elements may be lost during tumor development of adult cancers as opposed to pediatric cancers.

INTRODUCTION

The development of neoplasia in human cells appears to involve a number of genetic alterations including the activation of endogenous oncogenes and a loss of normal genetic information (1, 2). While the presence of altered oncogene expression has been well documented in human tumor cells, the concept of a loss of normal genetic elements in the development of human cancers is based on evidence which has been inferred from several types of studies. Several pediatric cancers have a rare hereditary form where deletions in specific chromosomes have been associated with the etiology of the disease. Examples include the deletion in chromosome 13q14 observed in retinoblastoma (3) and the deletion in chromosome 11p13 that is seen in Wilms’ tumors (4). A deletion in the p arm of chromosome 1 in neuroblastomas has also been observed, although a hereditary form has not been reported (5). It would appear that the loss of normal genetic information present in these deletions is associated with the malignant state.

Another series of studies supporting a loss of genetic information in the development of human cancer involves the characterization of somatic cell hybrids. Somatic cell hybrids between human tumor cell lines and normal human cells are initially suppressed for tumorigenic potential (6–9). Upon continued passage in culture, tumorigenic variants appear which have lost specific chromosomes. In the case of HeLa × normal human fibroblasts, copies of both chromosomes 11 and 14 were lost upon reexpression of tumorigenic potential (10, 11). In hybrids between HT1080 and normal human fibroblasts, loss of chromosomes 1 and 4 was associated with reexpression of tumorigenicity in the hybrid cells (8). An analysis of RFLPs in the HeLa × fibroblast hybrids showed that only the chromosome 11 from the normal fibroblast parent was lost upon reexpression of tumorigenicity (11, 12). Thus, it appeared that normal genetic elements present on these chromosomes have either been lost from these tumor cells or are no longer functional.

Previous research in the area of somatic cell hybrids has primarily involved cell lines derived from adult tumors. Since cytogenetic and familial studies have implicated a loss of normal genetic information from pediatric cancers, it was of interest to determine the genetic behavior of tumorigenicity in these tumor cells (3–5). Therefore, a variety of cell hybrids between an adult carcinoma, HeLa, and pediatric cancer cell lines were isolated. Tumorigenicity has been shown to behave as a recessive genetic trait in HeLa cells, the putative tumor suppressor gene(s) being associated with chromosome 11 (13). HeLa was used to prevent problems of nonviability observed in hybrid cells between human tumor and normal human cells (14).

The data in this report demonstrate that tumorigenicity behaves as a recessive genetic trait in pediatric tumor cells. Furthermore, it appears that different genetic elements control tumorigenic expression in pediatric and adult human tumor cells. In addition, it is shown that the expression of the N-myc and c-myc oncogenes, whose overexpression has been associated with the development of several pediatric cancers, does not correlate with the tumorigenic potential of these hybrid cells. Finally, the expression of the sis oncogene, which codes for the PDGF, does not correlate with tumorigenicity in these cells.

MATERIALS AND METHODS

Parent and Hybrid Cell Lines. The human cell lines utilized in this study are listed in Table 1. All cell lines have been previously characterized and are consistent with the type of tumor from which they were derived. Cell lines were grown in either Dulbecco’s modified Eagle’s or RPMI 1640 supplemented with 10% fetal calf serum (J. R. Scientific, Woodland, CA). All cultures were routinely tested for Mycoplasma contamination by DAPI assay (15) and were negative. Production and selection of hybrids were accomplished by the HAT and ouabain selection method reported elsewhere (16). Various combinations were made possible by the use of the universal fuser cell line D98OR, derived from HeLa, which is both hypoxanthine phosphoribosyl transferase deficient and ouabain resistant (16).

Hybrid Analyses. Hybrid formation was confirmed by chromosome analysis. Chromosomes were prepared according to the method of Nelson-Rees et al., and at least 25 metaphase spreads were examined for each clone (17). In cases where chromosome number was not informative, hybrids were also confirmed by RFLP analyses using probes for specific chromosomes. DNA probes specific for chromosomes 11 and 13 were provided by Dr. E. Srivastan of the Department of Ophthalmology at Children’s Hospital of Los Angeles. RFLP analyses were performed as previously described (12).

Tumorigenicity Assays. Cell suspensions containing 2 × 10⁶ to 2 × 10⁷ cells/0.2 ml were injected s.c. into the central midline of congenitally athymic nu/nu mice. Retinoblastoma hybrids were also injected...
intraocularly at an inoculum of 1 × 10^3 cells/eye. Mice were examined for tumors weekly, and tumor production was considered negative after 6 mo. Tumors formed by inoculation of hybrid cells were processed for histological examination and reconstituted into cell culture for chromosomal content.

**Oncogene Expression.** Oncogene expression was determined by Northern blot analysis. Total cellular RNA was purified by the guanidine hydrochloride extraction method (18). RNAs were fractionated by 1% agarose gel electrophoresis in the presence of formaldehyde and transferred to Biodyne hybridization filters. Appropriate cDNA probes were radiolabeled by primer extension (19) and hybridized to filters using the conditions described by Wahl et al. (20). The presence of RNA on each filter was verified by hybridization to the probe for α-tubulin. All lanes were found to have comparable amounts of total RNA.

**RESULTS**

Tumorigenicity Studies. If tumorigenicity behaves as a recessive genetic trait in pediatric cancers, then hybrid cells between HeLa and various pediatric tumor cell lines will be suppressed for tumorigenicity, providing different genetic elements have been altered in the parental cell lines. Therefore, a series of hybrid cells between D98OR, a HeLa cell derivative, and five different types of pediatric cancer cell lines were isolated. Since a deletion in chromosome 11 has been implicated in the development of Wilms’ tumors, it was of interest to determine whether HeLa and Wilms’ tumor shared a common genetic defect.

Table 2 summarizes the hybrid cell characteristics. Three separate fusions between the adult cervical carcinoma D98OR and two Wilms’ tumor lines (G401, SK-NEP-1) were examined. With one exception, all hybrids were suppressed for tumorigenicity (HHy14, HHy15, HHy16). The one exception, hybrid HHy15P3, had lost about 25% of the total chromosomes expected in a hybrid cell. Therefore, the genetic elements that were necessary for complete suppression of tumorigenicity observed in the other hybrids may not have been present in this cell line. These data indicate that tumorigenicity behaves as a recessive genetic trait in Wilms’ tumor cell lines. Furthermore, a different genetic element that controls tumorigenic potential is altered in the adult cervical carcinoma cell line than in the two Wilms’ tumor cell lines. Fusions between D98OR and four other pediatric cancers, a rhabdomyosarcoma (HHy30), a neuroblastoma (HHy23), an osteosarcoma (HOH), and a retinoblastoma (HHy17), also resulted in suppression of tumorigenicity. Thus, these other types of pediatric cancers contained genetic elements that were capable of suppressing the tumorigenic potential of HeLa cells.

If the suppression of tumorigenicity in these cell hybrids were due to the presence of “tumor suppressor” genes, then the loss of these elements should result in the reexpression of tumorigenic potential. While most human intraspecific hybrid cells are chromosomally stable, there is a certain amount of variance among hybrid cell populations. This was particularly true for the HeLa × 79 hybrid cells (HHy17). Tumors arose in some of the animals after the initial inoculation. When these tumors were reconstituted into tissue culture, there was a loss of approximately 10 chromosomes from the original hybrid cell population. A tumorigenic segregant of the 11 H\^23 hybrid had approximately 10 chromosomes from the original hybrid cell population. A tumorigenic segregant of the HHy23 hybrid had also undergone a significant reduction in chromosome number (data not shown). While these data do not address which chromosomes have been lost from the hybrid cells upon reexpression of tumorigenic potential, they are consistent with the loss of a “tumor suppressor” gene from these cells.

**Oncogene Studies.** It has been suggested that tumor suppressor genes might actually be "antioncogenes," genes which regulate the expression of known protooncogenes in cells. Therefore it was of interest to determine whether the expression of several oncogenes which have been associated with the parental pediatric tumor cell lines correlated with tumorigenic potential in the hybrid cells. Amplification of the N-myc oncogene has been shown in both the LA-N-5 neuroblastoma cell line and the Y79 retinoblastoma cells (21, 22). We examined the expression of N-myc mRNA in the nonmalignant D98OR × LA-N-5 (HHy23) hybrid cells (Fig. 1). While there is a high level of N-myc mRNA in the LA-N-5 parental cell, little or no expression was observed in either of the suppressed hybrid cells. Similar results were also seen for the D98OR × Y79 hybrid cells (HHy17) (Fig. 1). The loss of expression of this oncogene was due to a reduction of the structural gene to single copy number (data not shown). Therefore it initially appeared that the expres-
in the Y79 × HeLa hybrid cells. However, this change was not observed in the other two hybrid cells. Thus, the level of expression of the c-myc oncogene did not correlate with tumorigenic potential in the hybrid cells.

Expression of the sis/PDGF-2 oncogene has been observed in many human tumor cells and has been associated with the production of a growth factor activity (25, 26). The Wilms’ tumor cell line, G401, expressed detectable levels of mRNA for this oncogene. Expression of the sis/PDGF-2 oncogene has been observed in another kidney carcinoma (27). We therefore felt it would be of interest to determine whether any correlation existed between tumorigenic potential and the expression of this oncogene. Fig. 3 shows the expression of the sis oncogene in this set of cell hybrids. The HeLa × Wilms’ tumor hybrids showed variable levels of expression of sis as compared to the Wilms’ tumor parent; HHy15P1 showed no expression of the sis oncogene, while HHy14 showed the same levels as the G401 parent. A third clone HHy15P3 showed a somewhat reduced level of mRNA when compared to the parent. Since HHy15P1 and HHy14 are both nontumorigenic cell lines, tumorigenicity did not correlate with sis oncogene expression in these cells.

**DISCUSSION**

While previous studies from several laboratories have demonstrated that tumorigenic behavior as a recessive genetic trait in tumor cells derived from adult cancers, the genetic behavior of tumorigenicity in pediatric cancers had not been investigated. The importance of determining this was underscored by the fact that much of the evidence which supported the existence of recessive cancer genes came from familial and cytogenetic studies of pediatric cancers. The data in this report demonstrate that tumorigenicity behaves as a recessive genetic trait in cell lines derived from a variety of pediatric cancers. Thus, the cells have presumably lost genetic material which is involved in the expression of the tumorigenic phenotype. These results raise obvious questions about the association between “tumor suppressor” genes and chromosomal deletions that have been associated with certain pediatric cancers.

Saxon et al. and Weissman et al. have recently reported that the introduction of a normal human chromosome 11 into both HeLa and Wilms’ tumor cell lines suppresses their tumorigenic potential (13, 24). Therefore, the possibility arose that the same

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*Fig. 1. Expression of the N-myc oncogene in parental and hybrid cells. The expression of the N-myc oncogene in the hybrid cell lines between HeLa (D98OR) and a neuroblastoma (LA-N-5) and a retinoblastoma (Y79) is shown. The expression was determined by Northern blotting techniques as described in “Materials and Methods.” Each lane contains 40 μg of total cellular RNA. HHy17P2Tu0 is a tumorigenic segregant derived from the D98OR × Y79 hybrid cell line HHy17P2. To confirm equal loading of RNA, the filter was subsequently hybridized with a probe for α-tubulin (lower portion of figure). kb, kilobase.

*Fig. 2. Expression of the c-myc oncogene in the parental and hybrid cell lines. The expression of the c-myc oncogene was determined by Northern blotting techniques as described in “Materials and Methods.” The parental cell lines HeLa (D98OR), two Wilms’ tumor cell lines (G401 and SK-Nep-1), and the hybrid cells are shown. Each lane contains 40 μg of total cellular RNA. kb, kilobase.

*Fig. 3. Expression of the sis oncogene in the parental and hybrid cell lines. The expression of the sis oncogene was determined by Northern blotting techniques as outlined in “Materials and Methods.” Expression in hybrid cells between HeLa (D98OR) and a Wilms’ tumor cell line (G401) is shown. Each lane contains 40 μg of total cellular RNA. G401.2, a G401 cell line into which a normal human chromosome 11 has been introduced by microcell transfer, has the same level of N-myc mRNA. HHy17P2Tu0 is a tumorigenic segregant derived from the D98OR × Y79 hybrid cell line HHy17P2. To confirm equal loading of RNA, the filter was subsequently hybridized with a probe for α-tubulin (lower portion of figure). kb, kilobase.
genetic element on chromosome 11 that is lost during the development of Wilms' tumor is also absent in HeLa cells. However, the data in this study clearly demonstrate that hybrid cells between two different Wilms' tumor cell lines and a HeLa cell line are suppressed for tumorigenic potential. This result implies that Wilms' tumor cells and HeLa cells contain different genetic lesions which are involved in the control of tumorigenic potential. Furthermore, because existing data would suggest that Wilms' tumor cells contain two copies of a genetic defect at chromosome 11p13, these results would suggest that this site is probably not the location of the HeLa suppressor gene.

Recently a cDNA clone has been identified as coding for the putative retinoblastoma (rb-1) gene (28–30). The evidence for this conclusion comes from the mapping of this sequence to the area of chromosome 13q14 as well as the absence of the mRNA in retinoblastomas and osteosarcomas. The data from these cell hybridization experiments indicate that tumorigenicty can be suppressed in a retinoblastoma cell line (Y79) as well as an osteosarcoma cell line (OHS P16T) by the addition of presumably normal genetic material. Both of these cell lines have been shown to have altered expression of the rb-1 gene (28–30). It will be important to determine whether the addition of the normal rb-1 gene to these cells will result in the abrogation of their tumorigenic potential.

While the existence of recessive cancer genes rests on circumstantial evidence, oncogenes have been well characterized. Surprisingly, little information has been reported on the expression of oncogenes in somatic cell hybrids. Hybrids between normal human fibroblasts and two tumor lines having activated ras genes (8, 9) were totally suppressed for tumorigenic potential yet continued to express the M, 21,000 protein. The influence of other oncogenes on the tumorigenicity of hybrids has not been reported.

Altered expression of the N-myc oncogene has been associated with several types of pediatric cancers. When either a neuroblastoma cell line (LA-N-5) or a retinoblastoma cell line (Y79) which contained amplified copies of the N-myc oncogene was fused to a HeLa cell derivative which did not express N-myc, the hybrid cells were suppressed for tumorigenic potential. In a concomitant manner, N-myc expression was also suppressed, due to the loss of amplification of the N-myc oncogene in the hybrid cells. Since all hybrid cells arose from independent fusion events, these results imply a strong selective pressure against the amplification of the N-myc oncogene in the hybrid cells. When tumorigenicity was reexpressed in the HeLa × Y79 hybrid cells, there was no increase in the levels of N-myc mRNA. This result rules out the necessity for overexpression of the N-myc oncogene in order for tumorigenic potential to be reexpressed.

When the expression of the c-myc oncogene was examined in the HeLa × Wilms' tumor hybrid cells, no correlation of expression with tumorigenic potential was observed, although all parental cells expressed detectable levels of this gene. An examination of the amount of c-myc protein in the hybrid cells will also be required to confirm phase results. It has been proposed that N-myc and c-myc may control each other's expression, making it unlikely that both oncogenes will be expressed in the cell line (23). This might explain the strong selection against the expression of the N-myc oncogene in the retinoblastoma and neuroblastoma × HeLa hybrid cells. We are currently pursuing this question.

Autocrine production of growth factors has been suggested to play a role in tumor development. Production of PDGF-like factors by cells which overexpress the sis oncogene may be one example of this mechanism. Expression of the sis oncogene did not correlate with tumorigenic potential in hybrid cells between a Wilms' tumor cell line and HeLa. In two hybrid cells, the level of expression of the sis oncogene was similar to that observed in the parental cell line. This result agrees with previous data on microcell hybrids with the same Wilms' tumor cell line and human chromosomes t(X;11) and t(X;13) (24). However, it is interesting to note that, in one hybrid cell line, sis expression was not observed. Whether this is due to a loss of an activating factor or the addition of a regulatory gene(s) is currently being investigated.

The formal proof of the existence of recessive cancer and/or tumor suppressor genes will require their isolation by molecular biological techniques. The data in this report, as well as in a previous study, would suggest the existence of multiple tumor suppressor genes in the human genome (31). Introduction of individual chromosomes by microcell hybridization may further define the number of different genes which are responsible for the control of tumorigenic expression in cancer cells (13, 24, 32). However, the hybrid cell lines generated in this study will be useful in identifying protein or oncogene changes which correlate with tumorigenicity. In this manner, valuable information about the functions or products of putative recessive cancer genes may be gained.

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