Age-dependent Regulation of the Tumorigenic Potential of Neoplastically Transformed Rat Liver Epithelial Cells by the Liver Microenvironment

Karen D. McCullough, William B. Coleman, Gary J. Smith, and Joe W. Grisham

Department of Pathology and Curriculum in Toxicology, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Abstract

Neoplastically transformed rat liver epithelial cell lines (GN6TF and GP7TB), which form tumors with short latency at s.c. or i.p. transplantation sites of syngeneic rats, did not form tumors or were weakly tumorigenic following transplantation into the livers of young adult rats and expressed increasing tumorigenicity in livers of increasingly aged rats. These results suggest that progressive alterations in the hepatic parenchyma with increasing age enabled tumor formation by providing a less suppressive microenvironment for expression of the tumorigenic phenotype. Age is widely recognized as a significant risk factor in the development of neoplasia; this study describes a model for investigation of the influence of age-dependent changes in the hepatic microenvironment on the development of hepatic cancer.

Introduction

Age is the most important risk factor in the development of many types of cancer (1, 2). Despite the strong correlation between increasing age and cancer incidence, the mechanism underlying this relationship remains unknown. Nonetheless, two major hypotheses have been proposed to explain the dependence of cancer incidence on age (2). One hypothesis states that increased cancer susceptibility with advanced age may be related to the time required for accumulation of a sufficient number of genetic alterations to achieve neoplastic transformation in a target cell population (2). Peto et al. (3, 4) have proposed that advanced age at the onset of neoplasia simply reflects the duration of exposure to carcinogenic stimuli and that cancer incidence increases as a power function of this duration rather than as a power function of the age of the host. The second hypothesis proposes that progressive changes in the biological milieu accompanying increasing age may provide an increasingly favorable environment for the induction of new neoplasms and the expression of already extant but latent cancer (5). Age-related changes in metabolism, DNA repair, immune status (6), or the capacity of the tissue microenvironment to regulate cellular proliferation and differentiation could lead to the formation of a tumor late in the lifespan of the organism (7). Thus, the importance of age as a risk factor in the development of some human cancers may be related to both the accumulation of genetic damage with the passage of time and to age-dependent alterations of the tissue microenvironment, permitting the proliferation of neoplastic cells leading to tumor formation.

Previous studies investigating the relationship between age and tumorigenesis could not distinguish the effects of the aging tissue microenvironment and duration of carcinogen exposure (3, 8). Thus, the frequency of tumor formation in these investigations may have been affected by the aging of the host in coordination with the accumulation of genetic damage in response to long-term carcinogen treatment (6). Therefore, to address directly the effect of age on the ability of tumorigenic cells to form tumors in specific tissue microenvironments, we have used a model system that segregates the processes of carcinogen-induced neoplastic transformation and aging. This model system uses established lines of highly tumorigenic rat liver epithelial cells for transplantation into the livers of age-controlled syngeneic rats. We have shown previously that the tumorigenicity of some of these transformed lines (but not others) is suppressed when they are transplanted into the livers of young syngeneic rats (9). Suppression appears to involve the differentiation of transplanted cells into hepatocyte-like cells (9). Using this system, we have tested directly the effects of aging on the ability of the hepatic microenvironment to suppress tumor formation. The results of this study demonstrate that tumor incidence increases with the increasing age of the host, suggesting that the suppressive (and regulatory) potency of the hepatic tissue microenvironment declines with increasing age.

Materials and Methods

Cell Lines and Culture. The cell lines used in these studies were derived from a normal rat liver epithelial stem-like cell line termed WB-F344 that was isolated from an adult male Fischer 344 rat (10). WB-F344 cells were neoplastically transformed in vitro by treatment with N-methyl-N'-nitro-N-nitrosoguanidine, giving rise to clonal cell lines that expressed multiple transformation-associated phenotypes, including tumorigenicity (11). Tumor cell lines which expressed altered phenotypic properties were established from tumors which formed in syngeneic animals after transplantation of the chemically transformed cell lines (12). Two of these tumor cell lines, GN6TF and GP7TB, were used in the present studies. These tumor cell lines produce undifferentiated spindle cell carcinomas at s.c. or i.p. transplantation sites of 100% of syngeneic rats after a short latency period (9). The WB-F344 cells and tumor cell lines were maintained in Richter's improved minimal essential medium with zinc option supplemented with insulin (Irvine Scientific, Santa Ana, CA), as described (11).

WB-F344 cells and tumor cell lines (GN6TF and GP7TB) were genetically modified by infection with the defective CRE BAG2 retrovirus (13). The modified cells (named BAG2-WB, BAG2-GN6TF, and BAG2-GP7TB) express the retrovirally transected Escherichia coli β-galactosidase reporter gene and the neomycin (G418) resistance gene (9). Clonal sublines selected initially for G418 resistance were screened for β-galactosidase expression, and sublines expressing high levels of the marker enzyme were used for transplantation experiments. Infection of the tumor cell lines with the CRE BAG2 retrovirus did not alter their cellular phenotype or tumorigenic potential following s.c. transplantation (9).

Syngeneic Animal Hosts and Cell Transplantation. Age-controlled virgin male Fischer 344 rats were obtained from the Aged Rat Colony of the National Institute on Aging (Harlan Sprague Dawley, Indianapolis, IN). Animals at 3, 9, 12, 18, and 24 months of age were used in transplantation experiments within 7 days after receiving them. All animal studies were carried out in compliance with the guidelines established by the University of North Carolina at Chapel Hill Animal Care Committee.
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Out under the guidelines of the NIH and the Institutional Animal Care and Use Committee of the University of North Carolina.

Cells used for transplantation (BAG2-WB, BAG2-GN6TF, or BAG2-GP7TB) were trypsinized, washed in three changes of ice-cold Thilly’s buffered salt solution to remove traces of serum, resuspended in ice-cold Thilly’s buffered salt solution, and held on ice until used. Transplantation of cells was accomplished by direct transcapsular injection into the median liver lobe of rats anesthetized lightly with ether (9). Either 5 x 10^6 cells/0.2 ml (BAG2-WB and BAG2-GN6TF) or 1 x 10^6 cells/0.1 ml (BAG2-GP7TB) were transplanted into the livers of recipient rats. To insure uniformity, rats representing each age group received, on the same day, cell transplants from a single population of experimental cells. Age-matched control animals that were not surgically manipulated were maintained in parallel with rats that received cell transplants. Control rats and rats that received BAG2-WB cell transplants were euthanized at the end of 3 months (approximately 85 days). Rats that received BAG2-GN6TF or BAG2-GP7TB cell transplants were euthanized after 3 months or when moribund due to the development of tumors.

β-Galactosidase Histochemistry and Histological Evaluation. Harvested liver tissue was inspected for the presence of macroscopic tumor nodules prior to gross sectioning and further processing of representative portions of the tissues for histochemical detection of β-galactosidase and for histopathological analysis. Liver and tumor tissues intended for histological evaluation were fixed in formalin, embedded in paraffin, and sectioned and stained with hematoxylin and eosin according to standard procedures. Cryosections were prepared from tissues intended for histochemical analysis that had been fixed in situ by perfusion with 2% paraformaldehyde or directly frozen (9, 14). Tissue cryosections were postfixed in an ice-cold solution of 1% glutaraldehyde containing 100 mM NaPO₄ (pH 7.0) and 1.0 mM MgCl₂ for 15 min, rinsed briefly in a buffer containing 100 mM NaPO₄ (pH 7.0)-100 mM NaCl-5.0 mM MgCl₂, and incubated at 37°C in an X-Gal (100 mM NaPO₄ (pH 7.0), 150 mM NaCl, 1.0 mM MgCl₂, 3.3 mM K₃Fe(CN)₆, 3.3 mM K₄Fe(CN)₆, and 0.2 mg/ml 5-bromo-4-chloro-3-indoly β-D-galactopyranoside) substrate modified from that of Sanes et al. (15). The X-Gal substrate was dissolved in N,N’-dimethylformamide at 20 mg/ml and then diluted into the reaction mixture.

Results

During the 3-month study, no tumors formed in the livers of unmanipulated control rats of any age group or in rats of any age group that received BAG2-WB cell transplants (Table 1). Thus, in this study, spontaneous or trauma-related liver tumors were not found. Histochemical analysis of liver tissue from rats into which BAG2-WB cells were transplanted revealed numerous β-galactosidase-positive cells integrated into the hepatic plates among the host hepatocytes (data not shown). The β-galactosidase-positive cells were significantly larger than parental BAG2-WB cells and morphologically resembled mature hepatocytes. Hepatocytes in liver cryosections from control rats exhibited negligible background staining for β-galactosidase under the conditions used for histochemical detection (9, 14). These results show that the hepatic parenchyma induced the morphological differentiation of transplanted BAG2-WB cells in rats of all ages, consistent with our earlier studies that demonstrated morphological and functional differentiation of BAG2-WB cells following their transplantation into the livers of young adult rats (9, 14).

When transplanted into the liver, BAG2-GP7TB cells formed hepatic tumors in rats in each of the age groups examined (Table 1). The percentage of rats in which liver tumors formed increased with the increasing age of the hosts, reaching 100% in the 18-month-old age group. In addition, the majority of rats that received BAG2-GP7TB cell transplants developed extrahepatic tumors in the peritoneal cavity (12 of 14; 86%) as a result of leakage of cells from the injection site in the liver during the transplantation procedure, and several developed tumors in the lungs (3 of 14; 21%) from cells that escaped the liver via the circulation. BAG2-GP7TB-derived liver tumors were poorly differentiated in both young and old animals, morphologically resembling the s.c. or i.p. tumors produced by these cells (9). The latency for intrahepatic tumor formation was longest for the single tumor that arose in a rat of the 3-month age group, whereas tumor latencies were comparable for animals that received tumor cell transplants at 9, 12, and 18 months of age (49 ± 4 days; n = 8). These results suggest that the hepatic microenvironment of young rats (3 months old) can impose a small degree of regulation over the proliferation of transplanted BAG2-GP7TB cells, resulting in decreased tumorigenicity and increased latency for tumor formation. However, this regulation is dependent upon the number of cells transplanted since previous studies demonstrated that BAG2-GP7TB cells form tumors in livers of 100% of young adult rats when 5 x 10⁶ cells were transplanted (9).

In contrast to the minimal attenuation of growth observed with BAG2-GP7TB cells transplanted into the livers of young rats, the proliferation and tumorigenicity of BAG2-GN6TF cells was extensively regulated by the hepatic microenvironment of young adult rats. Microscopic inspection of cryosections representing different portions of the median liver lobe of young adult rats revealed no tumor nodules in any liver section. Thus, BAG2-GN6TF cells were not tumorigenic in the livers of 3- or 9-month-old rats (Table 1), even though 5-fold more cells were transplanted than for BAG2-GP7TB. Instead, individual β-galactosidase-positive cells were identified in all liver cryosections from each of the 3- and 9-month-old rats. The transplanted BAG2-GN6TF cells were integrated into the hepatic plates among host hepatocytes and morphologically resembled the adjacent mature hepatocytes (Fig. 1). However, in marked contrast to the absence of tumors in the liver in the 3- and 9-month-old age groups, extrahepatic tumors developed in the peritoneal cavities of 50% (8 of 16) of the rats as a result of leakage of cells from the injection site. In addition, some of these young adult rats (3 of 16; 19%) developed tumors in the lungs from transplanted BAG2-GN6TF cells that passed from the liver through the circulation. The expression of tumorigenicity by BAG2-GN6TF cells that gained access to the extrahepatic microenvironment suggests that the suppression of tumorigenicity by these cells within the liver is the direct result of interactions between the transplanted cells and factors contained in the hepatic microenvironment.

Table 1 Tumorigenicity of transplanted normal and neoplastically transformed rat liver epithelial cells

<table>
<thead>
<tr>
<th>Host age</th>
<th>Hepatic tumors</th>
<th>Latency a (days)</th>
<th>Hepatic tumors</th>
<th>Latency a (days)</th>
<th>Hepatic tumors</th>
<th>Latency a (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 Months</td>
<td>0/4</td>
<td>–</td>
<td>1/4</td>
<td>81</td>
<td>0/8</td>
<td>–</td>
</tr>
<tr>
<td>9 Months</td>
<td>0/4</td>
<td>–</td>
<td>2/3</td>
<td>44 ± 1</td>
<td>0/8</td>
<td>–</td>
</tr>
<tr>
<td>12 Months</td>
<td>0/4</td>
<td>–</td>
<td>2/3</td>
<td>53 ± 8</td>
<td>2/11</td>
<td>70 ± 4</td>
</tr>
<tr>
<td>18 Months</td>
<td>0/4</td>
<td>–</td>
<td>4/4</td>
<td>50 ± 4</td>
<td>5/8</td>
<td>65 ± 6</td>
</tr>
<tr>
<td>24 Months</td>
<td>0/3</td>
<td>–</td>
<td>ND b</td>
<td>–</td>
<td>8/8</td>
<td>40 ± 3</td>
</tr>
</tbody>
</table>

a Mean time to the detection and harvest of hepatic tumors. In some cases rats were euthanized due to extrahepatic tumor burden. Thus, this value represents the survival time for rats that developed intrahepatic tumors. Survival times for rats that did not develop hepatic tumors ranged from 32–85 days (80 ± 7 days for BAG2-GN6TF transplanted rats, n = 28; 85 ± 1 days for BAG2-GP7TB transplanted rats, n = 5). Animals that survived the duration of the study without tumors (including all BAG2-WB transplanted rats) were euthanized 85 days after transplantation.

b ND, not done.
TUMOR SUPPRESSION IN THE AGING LIVER MICROENVIRONMENT

The ability of the hepatic microenvironment to regulate the neoplastic phenotype of transplanted BAG2-GN6TF cells decreased with the increasing age of the host. In some older animals (12–24-month age groups) foci of β-galactosidase-positive hepatocyte-like cells were observed that formed plates of cells with sinusoidal spaces (Fig. 1). These foci of β-galactosidase-positive cells were connected to the hepatic plates of the host parenchyma at their periphery but disrupted the normal lobular organization of the host parenchyma. Such groups of β-galactosidase-positive hepatocyte-like cells were observed in older animals, both in the absence of identifiable tumor nodules and in the presence of tumors, but such foci were never observed in young adult rats (3- and 9-month age groups). The appearance of focal outgrowths of the transplanted BAG2-GN6TF cells within the parenchyma of older rats suggests that the strong regulation of cell proliferation apparent in the liver microenvironment in young rats (3–9 months old) diminishes in older rats (12–24 months old).

The percentage of rats in which liver tumors formed following transplantation of BAG2-GN6TF cells increased with increasing age for animals between 12 and 24 months old (Fig. 2), reaching 100% in the oldest rats (Table 1). The latency for tumor formation in the hepatic parenchyma by BAG2-GN6TF cells decreased, and the average tumorigenicity increased in progressively older animals (Table 1). Extrahepatic tumors formed in the peritoneal cavities of the majority of rats in the 12–24-month-old age groups (17 of 27; 63%) and in the

Fig. 1. Cryosections demonstrating the presence of transplanted BAG2-GN6TF cells in the hepatic parenchyma of rats of various ages. Liver cryosections were postfixed with glutaraldehyde, histochemical stained by reaction with an X-Gal substrate (12–16 h), and counterstained in Mayer's hematoxylin solution. A and B, liver sections from young adult rats demonstrating the presence of β-galactosidase-positive hepatocyte-like cells (3 months following cell transplantation). C and D, liver sections demonstrating focal proliferation of BAG2-GN6TF cells following transplantation into the livers of 12 month-old rats (3 months following transplantation). E and F, histological appearance of BAG2-GN6TF-derived tumors in the hepatic parenchyma of 24-month-old rats (42 days following transplantation).
Tumorigenicity is expressed as the percentage of rats developing liver tumors following transplantation with either BAG2-GN6TF or BAG2-GP7TB. Host rat age is given in months and represents the age of the animal at the time of transplantation of tumor cells. The tumorigenic liver epithelial cells used in these studies formed lungs of a fraction of these animals (6 of 27; 22%). BAG2-GN6TF-derived tumors which formed at both hepatic (Fig. 1) and extraparenchymal (i.p. or lung) sites were poorly differentiated, irrespective of the age of the host animal (Fig. 1). These data suggest that the hepatic parenchyma of young rats (3—9 months old) provides a strong stimulus for differentiation of BAG2-GN6TF cells, suppressing the neoplastic phenotype, but that with increasing age the stimulus for differentiation weakens, allowing cell proliferation and formation of foci and/or tumors in older rats (12—24 months old).

Discussion

The long latency period for the carcinogenic process in vivo is a general feature of neoplasia and may reflect the time required for genotypic evolution of carcinogen-altered cells and the accumulation of the phenotypic traits associated with autonomous growth (16, 17). However, the results of the present study suggest strongly that the tissue microenvironment represents an equally important determinant in the age-related tumorigenic potential of transformed epithelial cells. Our results demonstrated clearly that the liver microenvironment of young adult rats can regulate the differentiation of highly tumorigenic liver epithelial cells, coordinately suppressing the proliferation of the neoplastic cells. This observation suggests that the mechanism for tumor suppression by the liver involves hepatocytic differentiation of the neoplastic cells under the control of transactivating factors contained in the hepatic microenvironment. Numerous reports in the literature have demonstrated that the interaction of neoplastic cells with the microenvironment of the developing embryo can result in both suppression of tumorigenic potential and cellular differentiation of the transformed cells (18—22).

The tumorigenic liver epithelial cells used in these studies formed tumors with increasing frequency in progressively aged rats, suggesting that older rats have sustained an age-related loss or modification of some critical microenvironmental signal that is required for normal regulation of cell proliferation and differentiation. In addition, as demonstrated by comparing BAG2-GN6TF and BAG2-GP7TB, the degree of phenotypic evolution influences the response of the transplanted cell population to hepatic regulatory signals. Thus, neoplastic lineages that express highly transformed phenotypes may rapidly escape the regulatory constraints of the liver microenvironment, even in young adult animals in which the suppressive influences are the most potent. A less potent transformation-associated cell phenotype would be associated with a more indolent course, which would be accelerated by acquisition of additional mutational or epigenetic changes in gene expression. Hence, the process of hepatocarcinogenesis in vivo may include rate-limiting steps for (a) evolution of phenotypic changes in the altered cell population (development of tumorigenic potential) and (b) age-related alteration of tumorigenic potential by the liver microenvironment (development of an environment conducive for tumor formation). Consequently, both cellular and microenvironmental factors contribute to the expression of a neoplastic phenotype, suggesting that tumorigenic potential may be determined by the relative balance between the regulatory potency of the tissue microenvironment and the accumulated mutational/epigenetic alterations in the potentially tumorigenic cell lineage. Alteration, reduction, or elimination of critical signals necessary for the normal regulation of cell proliferation and differentiation by the tissue microenvironment could allow proliferation of latent populations of potentially tumorigenic cells, leading to the formation of a tumor.

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References

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