PRENEOPLASTIC LESION GROWTH IN B6.Hcs7C3H 219R8 CONGENIC HEPATOCARCINOGEN-SENSITIVE MUS MUSCULUS

The Hcs7 locus on mouse Chromosome 1 is involved in the increased susceptibility to liver carcinogenesis in C3H mice relative to the resistant B6 strain. Early experiments have suggested that Hcs7 affects the promotion phase of hepatic tumor development. Male C3H, B6, and B6 mice carrying the C3H Hcs7 allele were treated with DEN, sacrificed at two time points, and their livers dissected. Preneoplastic lesions in the liver sections were counted and measured. A computer program was used to extrapolate the volume fraction change of the lesions between the two time points to determine if the locus promotes liver tumorigenesis. Understanding the mechanisms of liver cancer in mice can allow the identification of genes that have the same effect in human liver cancer.

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COVER SHEET

TITLE: Preneoplastic Lesion Growth in B6.Hcs7C3H 219R8 Congenic Hepatocarcinogen-Sensitive *Mus musculus*

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Abstract

A region of mouse Chromosome 1, called the *Hcs7* locus, is involved in the susceptibility to liver carcinogenesis in the inbred mouse strain, C3H/HeJ (C3H). Linkage analysis of a cross between C3H and a strain that is resistant to carcinogen-induced liver cancer, C57BL6/J (B6), has mapped this locus to the distal end of the Chromosome 1. Several genes in this area could play a role in the increased susceptibility in C3H mice. Initially, however, it should be determined whether or not the locus is affecting the initiation of tumors or the promotion of tumor growth in C3H mice. Early experiments have suggested that *Hcs7* affects the promotion phases of hepatic tumors after the carcinogen treatment. By observing the number and size of preneoplastic lesions in C3H, B6, and in B6 mice carrying the C3H allele of *Hcs7*, it was determined that there is a correlation between the locus and an increased growth rate in preneoplastic lesions in the liver. Further understanding the mechanisms of liver cancer in mice can allow one to begin to question and test whether the same processes exist in human liver cancer.

Introduction

Cancer is a disease caused by the uncontrolled growth of cells. The common theory of carcinogenesis states that cancer is characterized by three stages: initiation, promotion, and progression. Initiation events include the acquisition and fixation of a mutation of a DNA base or sequence. Promotion involves the aberrant gene expression that results from those mutations, which alters the regulation of cell growth and cell death, and results in clonal expansion of these mutated cells. (Pitot, 2002). At this point,

the mutated cells are called preneoplastic lesions: they are not healthy cells, and they have the ability to become cancerous. If the body can not control the growth of these cells, the preneoplastic lesions enter the third stage, progression, and become malignant.

Cancer can occur in almost all tissues. When mutations and uncontrolled growth occur in hepatocytes, liver cancer can result. Liver cancer, which occurs mostly in men, can result from hereditary predispositions, bacterial and viral infections, cirrhosis, and smoking. In 2008, liver cancer caused nearly 20,000 deaths in the United States (www.cancer.gov). In laboratory research, *Mus musculus*, or mice, provide excellent models to study liver cancer. Several of the genes and processes that are mutated in cancer in mice are homologous to genes and processes in humans.

Previous experiments have shown that C3H mice are much more susceptible to spontaneous and chemically induced liver tumors than B6 mice (Hanigan *et al* 1988; Bilger *et al*. 2004). Spontaneously, close to half of all 2 year-old C3H mice will develop liver tumors. In comparison, less than 5% of 2 year-old B6 mice develop tumors (Storer 1966, Frith and Wiley 1982). When both inbred strains are treated with the chemical carcinogen *N*,*N*-diethylnitrosamine (DEN) at 12 days of age, C3H male mice develop 20-50 fold greater number of tumors than B6 male mice (Hanigan *et al* 1988). In addition to the greater number of tumors, preneoplastic hepatic lesions in C3H male mice grow at a rate that is 1.7 times faster than B6 male mice (Hanigan *et al* 1988).

The majority (~85%) of the difference between the response to chemical carcinogen between C3H and B6 mice is due to one locus (Drinkwater and Ginsler, 1986). Linkage analysis of the two inbred strains showed that there is a statistical correlation between susceptibility to liver tumors and C3H alleles on mouse chromosome

1("chr. 1"; Bilger *et al*. 2004). This locus on chr. 1, *Hcs7*, is near the distal end of the chromosome, in the area of microsatellite markers D1Mit285, and D1Mit33 (Bilger *et al*. 2004).

In order to test that this locus on Chr. 1 is involved in the increased susceptibility to liver tumors in C3H mice, recombinant mice were generated so that the mice had the entire B6 genome except for the C3H chr. 1. Then, B6 mice that were homozygous or heterozygous for different lengths of C3H regions on chr. 1, were treated with DEN at 12 days of age, and the number of tumors was counted at 32 weeks of age. From this experiment, a mapping analysis showed that there is a 6.5Mb region between 170Mb and the end of Chr. 1 (197Mb), which is correlated with most of the sensitivity to liver tumors. B6 recombinants that only had a portion of C3H DNA on Chr. 1 from 168 Mb to 177Mb were selected for, which resulted in the sensitive B6.C3H R8 line (Figure 1). These susceptible recombinants had an average of 35 tumors, while B6 mice usually have an average of 6.5 tumors (data from personal communication with A. Bilger).

	164Mb	169Mb	177Mb	178Mb	# Tumors	# Animals
R8	**B6**	**C3H**		**B6**	35.0	26
B6	**B6**				6.5	23

Figure 1: Representation of Chr. 1 congenic structure, and susceptibility data.

Now that the *Hcs7* locus on Chr. 1 has been localized to the distal end of the chromosome, it will be worthwhile to discover if this locus is involved in susceptibility to the initiation of mutated cells or the promotion of growth of preneoplastic foci in C3H mice. Data from previous experiments suggests that C3H mice might be more susceptible to the promotion of liver carcinogenesis. For example, when the B6 and C3H

strains are crossed, preneoplastic lesions in the F1 generation appear to grow faster and have a higher frequency of becoming malignant than those in the resistant B6 parental strain (Dragani *et al.* 1987, Becker 1982). Also, when the DNA in DEN-injected B6 and C3H mice was analyzed, it was determined that both strains had the same number of DNA adducts, which can be fixed to form mutations (Drinkwater and Ginsler, 1986). This suggests that both inbred strains are obtaining the same number of initiation events, but C3H are more susceptible to the promotion of the preneoplastic cells, which then results in a higher susceptibility to liver cancer.

In this experiment, the livers of the parental strains and of the congenic were observed at two different time points after DEN treatment, to determine if there is a statistical relationship between the *Hcs7* susceptibility loci and either the initiation of lesions or the promotion of liver lesion growth. After the mice received the DEN carcinogen treatment at 12 days of age, they were sacrificed at 16 weeks and 24 weeks of age and their livers were analyzed for glucose-6-phosphatase (G6P) deficient foci. Liver tumors are thought to be derived from lesions in the liver that no longer produce this enzyme (Hanigan *et al* 1988). If *Hcs7* is affecting the promotion of preneoplastic lesions in the livers of C3H mice, then the congenic lesions should show a faster growth rate over the two time points compared to B6 mice.

Results from a similar, previous experiment with a recombinant strain that had a larger portion of C3H DNA inserted into the B6 chr. 1 suggest that the C3H allele at *Hcs7* causes preneoplastic lesions to grow larger and faster. The data from this experiment uses a smaller congenic, and expanded upon these preliminary results.

Methods

Mice and Carcinogen Treatment

Three groups of mice were bred in the laboratory: B6 (group 1), C3H (group 2), and the heterozygous congenic B6.C3H R8, which will from here on be referred to as 219R8 (group 3). The homozygous control groups, B6 and C3H, were bred from stocks purchased from Jackson Laboratory (Bar Harbor, ME). The mice were housed in plastic cages on corncob bedding, and were checked daily and weighed monthly. The two inbred strains, C3H and B6, as well as the recombinant strain, 219R8, received DEN carcinogen treatment at 12 days of age. Twelve mice from each group were sacrificed by CO^2 asphyxiation at 16 weeks. Another 12 mice from each group were sacrificed at 24 weeks. The liver from each mouse was removed and weighed, and the spleens were removed for DNA prep and genotyping.

Preparation and Analysis of Liver Sections

For each mouse, four 4mm sections from the left lateral and median lobes of the liver were placed on a tissue block and frozen on dry ice for cryosectioning. Cryosectioning is a procedure for freezing the parts of the liver and slicing them into 10μm sections so that the cells can be viewed under a microscope. This process was done by the McArdle Histology Department. The liver slides were then stained for glucose-6-phosphatase-deficient loci. The stain used forms a brown lead precipitate when it reacts with G6Pase. Therefore, preneoplastic lesions that are not producing the enzyme appear tan or unstained under the microscope.

Each slide was viewed under a microscope with an ocular micrometer. The ocular micrometer contains a scale that allows for the measurements of objects viewed under the

microscope at different magnifications. The shortest and longest dimensions of each lesion were measured to determine the approximate area of each lesion. The area of each liver section was determined using ImageJ (http://www.rsbweb.nih.gov/ij/). These area measurements were then used to determine the fraction of the liver that is occupied by preneoplastic lesions.

Statistical Analysis

The lesion areas and the corresponding liver area of each slide were entered into a computer program, Mstat, which was developed by Dr. Norman Drinkwater. Mstat is able to perform a stereologic analysis on the information from the liver sections. The data collected under the microscope is planar. Mstat uses an algorithm to convert the lesion size and liver area into the volume fraction, or the amount of 3-dimensional space the preneoplastic lesions are taking up in the livers at the two different time points. We then used this information to observe the change in volume fraction over the two time points to determine if the congenic preneoplastic lesions were growing faster than those in B6.

Once the data were obtained, a Wilcoxon Rank Sum test was used to determine the statistical significance of differences in the average volume fraction between experimental groups.

Results

Mstat provided the mean volume fraction, foci/cubic centimeter (cc), and cells/focus for each group at each time point. The data is presented in Table 1. Volume fraction is the estimated amount of space occupied by preneoplastic lesions in the liver.

Foci/cc is the amount of lesions in one cubic centimeter of liver. Cells/focus is the estimated number of cells in each lesion.. Due to estimation, small sample size, and the high variance in the number and sizes of lesions among the animals in a group, the error for each measurement was relatively high.

	B6		C3H		219R8	
	16 Wk	24 Wk	16 Wk	24 Wk	16 Wk	24 Wk
N (animals)	13	12	12	11	12	12
Vol. Fraction	0.07%	0.42%	1.11%	13.10%	0.24%	1.35%
Foci/cc	84.68	123.38	165.6	465.95	207.75	155.05
Cells/Focus	123.97	7274.17	275.43	54843.02	2669.31	20566.14

Table 1: Volume Fraction (%), Foci/cc, and Cells/Focus for each group.

In order to determine if the volume fraction among the experimental groups differed significantly, a Wilcoxon rank sum test was used. Comparisons between the 219R8 congenic and B6 mice provide the most information about the difference in susceptibility that the Hcs7 locus is providing, so only the data comparing those two strains is shown in the following tables.

Sample	N	Mean	SD
B6 (A)	13	0.0007	0.0006
Congenic (A)	12	0.0024	0.0002
			p= 0.0016

Sample	N	Mean	SD
B6 (B)	12	0.0111	0.0414
Congenic (B)	12	0.0135	0.0119
			p= 0.0047

Tables 2A and B: Wilcoxon Rank Sum Test for Lesion Volume Fraction in B6 and Congenic mice, at 16 weeks (A) and 24 weeks (B).

As seen in Table 2, both of the Wilcoxon rank sum tests had a significant *p*-value. This suggests that congenic lesion volume fractions are statistically significantly larger

than the B6 lesion volume fraction at each time point. The values for volume fraction for C3H males were statistically different from both B6 and the congenic males at each time point.

To estimate the difference in the rate of growth between the two time points in B6 and congenic mice, the cells/focus data was used. These data give an estimate on the amount of growth between the two time points because it can reflect the number of divisions occurring in the lesions. Table 3 shows the fold difference in the number of cells per focus in B6 and congenic mice at each time point (bottom row) and between time points (last column).

	Cells/Focus		
	16 Wk	**24 Wk**	**Fold Difference**
B6	1748.9	7274.17	4.16
219R8	2669.31	20566.14	7.70
Fold Difference	1.53	2.83	

Table 3: Comparison of Cells/Focus data between B6 and Congenic mice.

The data presented in Table 3 support the hypothesis that lesions in the 219R8 congenic are growing faster than lesions in B6 mice. Over the two time points, the congenic had close to an 8-fold increase in the number of cells per focus, whereas B6 lesions showed a 4-fold increase. The number of cells per focus in the congenic was 1.5-fold greater than in B6 mice at 16 weeks, and then increased to about 3-fold greater at 24 weeks.

Discussion

It was hypothesized that the *Hcs7* locus on Chr. 1 increases the susceptibility to liver cancer in C3H mice during the promotion stage of carcinogenesis. In this experiment, a congenic B6 mouse with the C3H allele of *Hcs7* was used to determine the difference in growth of preneoplastic lesions compared to a B6 control, between 16 and 24 weeks after exposure to DEN.

The cells/focus data supports the hypothesis that the *Hcs7* locus causes an increase in growth rate of lesions. The number of cells in the preneoplastic lesions in congenic mice increased more than the number of cells in B6 mice (Table 3). This suggests that one or more C3H alleles in the *Hcs7* locus are causing the preneoplastic lesions to grow faster, and supports the hypothesis that this locus is involved in the promotion of liver carcinogenesis.

The purpose of choosing the two time points was to observe the growth of the preneoplastic lesions between 16 and 24 weeks after exposure to DEN. However, in concordance with the results of the Wilcoxon rank sum tests using volume fraction data, it is clear that the lesions in congenic mice are not only growing faster between the two time points, but they also show significant increased growth before 16 weeks. This suggests that the gene, or genes, in the *Hcs7* locus that are causing the increased susceptibility in C3H mice are showing an effect early in the promotion stage before 16 weeks. This effect is most likely carried over to the time between 16 and 24 weeks, where the congenic was shown to have a greater increase of cells/focus than the B6 control group.

Conclusion

From the results of this experiment, it can be inferred that the *Hcs7* locus most likely causes an increase in lesion growth rate in C3H mice. The next steps would be to pinpoint the exact time after DEN exposure that there is a difference in the expression of genes in the locus in C3H mice, and to then discover which specific gene or genes is responsible for this difference.

A recent development in cancer research is the role of the inflammatory response in cancer susceptibility. It is thought that cytokines and growth factors produced by leukocytes in response to infection and carcinogen damage can often play important roles in the promotion and progression of cancer (Karin and Greten 2005). For example, when DEN is injected into the livers of the mice at the beginning of this experiment, leukocytes would have built up an inflammatory response to the carcinogen. These cells that are trying to defend the body from the carcinogen might also play a direct or indirect role in the promotion of preneoplastic lesions.

Using a database such as Ensembl, one can search the genes that have been mapped to the region of chr. 1 where *Hcs7* is located. The chr. 1 region from 173Mb to 179Mb (the current minimal susceptibility region as determined using congenic mice; Andrea Bilger, personal communication) contains approximately 100 genes. Many of these genes are involved in the inflammatory response. These inflammatory genes include Slamf1, Aim 2, and ifi204 (ensembl.org). Aim2 is a gene that has already been characterized as a tumor suppressor. The gene's name stands for "absent in melanoma," because it was discovered to be missing in several melanoma patients. The protein produced by Aim2 expression regulates NF-κB, which is a transcription factor involved

in the inflammatory response (Woerner *et al.* 2007). Deletions or mutations of the gene have not yet been characterized in liver cancer.

Using mouse models to discover the processes involved in liver cancer might provide key ideas for the development of prevention and treatment programs. It has already been shown that taking adult-strength aspirin, a drug that reduces the inflammatory response, has been associated with a reduced incidence of several cancers, such as colon, prostate and breast cancer (Jacobs *et al.*, 2007). Further characterization of the inflammatory response's role in tumor progression might provide more robust prevention strategies in humans.

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