Transplantation System for Determining the Clonogenic Survival of Parenchymal Hepatocytes Exposed to Ionizing Radiation

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ABSTRACT

An in vivo transplantation technique has been developed which can be utilized to determine the reproductive survival of parenchymal hepatocytes exposed either in vitro or in vivo to both physical and chemical genotoxic agents. We have used this assay system to determine the survival of liver cells exposed to sparsely ionizing radiation. The D0 value of the survival curve was 249 rads and the extrapolation number was 1.2. These results indicate that hepatocytes irradiated while in the Go phase are unable to accumulate sublethal damage to an appreciable extent if they are stimulated to undergo replication within 24 hr after the infliction of the damage.

INTRODUCTION

Liver is frequently the organ of choice for studies of carcinogenesis for a number of reasons. Liver can activate a variety of procarcinogens; it responds to partial hepatectomy with an orchestrated wave of cell replication; and it responds to many exogenously administered chemicals with the formation of neoplasms. Thus, in the liver, one can compare normal and neoplastic growth. Numerous studies have shown that carcinogenesis in the liver is accompanied frequently by cell death due to toxicity and by changing cell subpopulations due to compensatory regenerative activity (10, 25, 33). Despite the extensive use of liver carcinogenesis as a model for the evaluation of carcinogenic agents, the reproductive survival of hepatocytes has not been precisely quantified because of the lack of a suitable biological in vitro or in vivo assay system. This is also true for many other epithelial tissues. As a result, the carcinogenic potency of carcinogens is empirically determined by consideration of the tumor yield, tumor latency, and carcinogenic dose as described by Iball (16). Transformation and mutagenesis studies with cell culture systems allow the estimation of the numbers of transformants or mutants on the basis of the survival clonogenic units rather than on the basis of the total number of cells exposed. In this report, we introduce a system in which the clonogenic survival of the hepatocytes can be accurately assayed by the use of a transplantation system of dispersed parenchymal hepatocytes.

We have used X-irradiation as the genotoxic agent because it can be readily and precisely administered. In addition, there exists an interest in the determination of the effects of X-rays on hepatocyte survival in clinically relevant situations. Although there is frequently metastatic tumor involvement in the liver, the ability to successfully treat these tumors with radiation alone or in combination with chemotherapeutic drugs is low. The effect of ionizing radiation on the functionality of the liver has been determined with 131I-rose bengal (19). The pathological alterations which occur after irradiation have been described (13, 29). The sensitivity of irradiated liver to interphase death has also been estimated (17). However, although the liver has a tremendous capacity to regenerate after exposure to genotoxic agents, the reproductive survival of radiation-exposed liver cells is presently unknown.

Because an estimate of the reproductive survival of parenchymal hepatocytes is necessary for quantifying carcinogenic risk and also for improving the therapy of both primary and metastatic liver tumors, we have developed a system which can be used to estimate the reproductive survival of hepatocytes exposed to a genotoxic agent. We have utilized it to generate the reproductive survival curve for parenchymal hepatocytes exposed in the Go phase to single doses of low-LET4 radiation.

MATERIALS AND METHODS

Animals. Female syngeneic rats (Fischer 344) weighing approximately 100 g were obtained from Charles River Breeding Laboratories (Wilmington, Mass.) and were used as hepatocyte donors and recipients. They were housed in a temperature-controlled room with a 12-hr light-dark cycle. Autoclavable Laboratory Chow 5010 (Ralston Purina Co., St. Louis, Mo.) and water were provided ad libitum.

Irradiation. The rats were placed in a circular plexiglass holder (diameter, 15 cm; height, 6 cm) prior to irradiation. The holder was placed in a temperature-controlled room with a 12-hr light-dark cycle. Autoclavable Laboratory Chow 5010 (Ralston Purina Co., St. Louis, Mo.) and water were provided ad libitum.

Hepatocyte Preparation and Transplantation. Within 30 min after irradiation, the hepatocytes were enzymatically dispersed by the in situ 2-step collagenase perfusion technique as described by Berry and Friend (1) and modified by Deschenes et al. (6). Briefly, the liver was first perfused through the vena cava with a calcium-free buffer (150 ml) at a rate of 12 ml/min. One liter of the buffer contained potassium chloride (0.5 g), sodium chloride (8.3 g), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (2.4 g). The pH of the buffer was adjusted to 7.4 with sodium hydroxide. After the conclusion of this initial perfusion, the buffer was changed to one of identical composition with the addition of calcium chloride (CaCl2·2H2O) at a concentration of 740 mg/liter and collagenase at a concentration of

1 Supported by NIH Research Grant 5RO1CA25951-02 from the National Cancer Institute.
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3 Supported by NIH Research Grant CA15704 from the National Cancer Institute.

Received March 17, 1981; accepted June 16, 1981.

4 The abbreviations used are: LET, linear energy transfer; LNDso, number of
the liver was removed and minced with a scissors. The resultant material was filtered through a nylon mesh filter (100-µm pore size) and washed 3 times with Leibowitz-15 (L15) medium by centrifugation (50 x g). Very little clumping of the cells was observed and the cellular yield was approximately 50%. Of the dispersed cells, 75% excluded trypan blue and were scored as morphologically viable.

The cell survival was estimated by an end point dilution assay technique as described by Hewitt and Wilson (15). Appropriate 2-fold serial dilutions of the dispersed hepatocytes were made in L15 medium (Grand Island Biological Co., Grand Island, N. Y.). Each dilution of cells was mixed with an equal volume of a 50% brain homogenate suspension. The brain homogenate (1:1, w/v) was made in L15 medium with the use of an all-glass hand-driven homogenizer. The contribution of the brain tissue in the final cellular suspension was 25% (w/v). A volume of 0.06 ml of each cellular suspension was injected with a Hamilton syringe into the interscapular and both anterior lateral fat pads of each recipient animal. All recipient animals were two-thirds hepatectomized, via the technique of Higgins and Anderson (14), 1 to 2 hr prior to hepatocyte injection. Twenty days after transplantation, the recipient animals were sacrificed, and the fat pads were removed, whole mounted on glass slides, and stained. The histological appearance of the transplantation site as a function of time after liver cell injection has been described previously (18).

Data Analyses. The stained fat pads were viewed under a stereo microscope (×80), and the percentage of the injection sites for each dilution which contained at least one liver nodule was determined. The LND50 value at each radiation dose was estimated by the method of maximum likelihood based on the statistical evaluation according to Finney (11) as modified by Porter et al. (24). Briefly, the model states that

\[ P(Z) = 1 - e^{-M} \]  

where

\[ \log M = \log K + \log \frac{Z}{S} \]

and \( P(Z) \) is the probability of liver nodule formation at the injection site when an average of \( Z \) morphologically viable hepatocytes are injected. \( M \) is the average number of clonogens, \( K \) is the clonogenic fraction, and \( S \) is the slope of the relationship. When \( S = 1.0 \), \( P(Z) \) is derived from a Poisson distribution for the number of liver nodules formed. The maximum likelihood estimates of \( S \) and \( K \) were used to calculate the LND50 value at each dose of radiation. A \( \chi^2 \) test was used to determine whether the LND50 values were significantly different from each other.

The fraction of hepatocytes which survived radiation exposure was determined by dividing the LND50 value for unexposed cells by that obtained after exposure. The multitarget-single-hit model (9)

\[ S(D) = 1 - (1 - e^{-D/D_0})^n \]

was used to describe the survival data. \( S(D) \) is the survival fraction at dose \( D, D_0 \) is the reciprocal of the terminal slope, and \( n \) is the ordinate extrapolation of the linear component of the survival curve. The \( D_0 \) and \( n \) values were estimated along with their 95% confidence intervals by the method of weighted least squares as described previously (3).

Use of Multiple Injection Sites. When more than 100,000 morphologically viable irradiated cells were injected into a single site, it became difficult to determine whether the presence of a liver nodule was due to the proliferation of a single surviving cell with unlimited reproductive capacity or to the aggregation of microcolonies which form from aborted proliferative attempts of reproductively dead cells (Fig. 2b). To effectively eliminate this problem, we never injected more than 100,000 irradiated cells into a single site. Thus, when we required more than this number of cells to increase the probability of observing a liver nodule to 1.0, rather than injecting more cells we used multiple sites of injection (e.g., 2 sites would equal 200,000 cells, etc.). The proof in the appendix demonstrates that this treatment is justified only if \( S = 1.0 \); i.e., the number of liver nodules formed is described by a Poisson distribution. In "Results," we demonstrate empirically that at all radiation doses the \( S \) value is not significantly different from 1. Thus, the multiple-injection site approach can be used for hepatocytes.

RESULTS

Fig. 1 is a whole-mounted fat pad 20 days after hepatocyte injection into the interscapular fat pad of a two-thirds hepatectomized syngeneic recipient. Three liver nodules are shown in the figure. Each nodule is composed of numerous hepatocytes which can be seen in Fig. 2a. In the assay system that we have developed, a transplantation site is scored as positive for liver nodule formation if at least one liver nodule is present.

The probability of liver nodule formation as a function of the number of injected unirradiated morphologically viable hepatocytes is shown in Chart 1. A \( \chi^2 \) goodness of fit test demonstrated that Model 1 adequately (\( \chi^2 = 11.54; d.f. = 16; p = 0.8 \)) described the data. The estimated value of \( s \) (0.9; 95% confidence interval, 0.7 to 1.1) was not significantly different (\( p > 0.1 \)) from 1. Thus, the hepatocyte transplantation data are adequately described by a Poisson distribution. The LND50 value for unexposed liver cells was 2100 cells (95% confidence interval, 1600 to 2700 cells).

Peters and Hewitt (23) demonstrated that, when malignant cells were mixed with an homogenate of brain prior to cellular injection, fewer cells were required to form tumors in 50% of the injection sites. For both transplanted mammary (12) and thyroid (5) epithelial cells, it was also found that the presence of brain homogenate improved the clonogenicity of the cells. When hepatocytes were not mixed with brain homogenate prior to their injection into two-thirds hepatectomized rats, the LND50 value was significantly increased from 2,100 to 11,400 cells (95% confidence interval, 7,900 to 25,500; \( p < 0.001 \)). The value of \( S \) was again not significantly (\( p = 0.1 \)) different from 1. Thus, brain homogenate similarly improved the clonogenicity of transplanted hepatocytes.

Revesz (26) demonstrated that, when radiation-sterilized tumor cells were added to viable cell inocula, the number of viable cells required to produce a tumor was reduced. However, the presence of radiation-sterilized mammary (12) and thyroid (5) epithelial cells did not affect the transplantability of the unexposed cells. When approximately \( 5 \times 10^4 \) lethally...
irradiated liver cells were added to the mixture of nonirradiated cells and brain homogenate, the LND$_{50}$ value was 3400 cells (95% confidence interval, 2400 to 4500). This was not significantly lower than that when the lethally irradiated cells were absent from the inocula. The shape of the curve was also not altered ($S = 1.1; 95\%$ confidence interval, 0.7 to 1.4). Thus, under the assay conditions utilized, the presence of radiation-sterilized hepatocytes at the high radiation doses will not artificially increase the calculated estimate of the surviving fraction.

Chart 2 demonstrates how the LND$_{50}$ values change as a function of the total dose of ionizing radiation received. The LND$_{50}$ values at 500, 750, and 1300 rads were 10,900 (95% confidence interval, 5,500 to 22,600), 45,400 (95% confidence interval, 22,400 to 67,800), and 265,300 cells (95% confidence interval, 179,200 to 481,600), respectively. The values of the slopes at these 3 radiation doses were 0.7 (95% confidence interval, 0.3 to 1.2), 1.2 (95% confidence interval, 0.4 to 1.9), and 1.0 (95% confidence interval, 0.5 to 1.6), respectively. At all doses, the value of $S$ was not significantly different from 1 ($p > 0.2$), and the $S$ values obtained for irradiated cells were not different from those for unirradiated cells ($p > 0.2$). Since the shape of the curve was not dependent upon the dose of radiation that the cells received, the surviving fraction after radiation exposure can be estimated by dividing the LND$_{50}$ value for unexposed cells by that for exposed cells (9).

On Chart 3, the probability of cell survival after radiation exposure is graphed versus the dose of radiation received. A lack of fit test and a plot of residuals demonstrated that the multitarget-single hit model (Equation C) adequately ($p > 0.10$) described the survival data. When only the data from 650 to 1300 rads were analyzed (i.e., the probability of survival $<0.10$), the estimated values of $n$ and $D_0$ were 0.8 (95% confidence interval, 0.4 to 1.8) and 277 rads (95% confidence interval, 226 to 359 rads), respectively. If all the data were included in the statistical analysis, $n = 1.2$ (95% confidence interval, 0.8 to 2.0) and $D_0 = 248$ rads (95% confidence interval, 217 to 289 rads). Thus, for parenchymal hepatocytes irradiated while in the G$_0$ phase, the $D_q$ value [$D_q = \log(n)D_0$] was not found to be significantly different from zero.

**DISCUSSION**

Recently, we have described the histochemical and morphological appearance of hepatocytes transplanted into the fat pads of syngeneic recipients (18). In this report, we have demonstrated how this transplantation method can be utilized for quantifying the probability of reproductive survival of parenchymal hepatocytes after exposure to genotoxic agents. In this study, hepatocytes from intact nonregenerating livers were exposed to single doses of low-LET radiation. The resulting survival curve has a $D_0$ value of 248 rads and an extrapolation number of 1.2. These results indicate that parenchymal hepatocytes exposed to radiation while in G$_0$ cannot accumulate sublethal damage to a significant extent if they are placed in conditions in which they are forced to replicate within 24 hr after exposure.

To estimate the reproductive survival of hepatocytes, we used the end point dilution assay technique. This procedure was originally utilized by Hewitt and Wilson (14) to determine the radiation survival of leukemia cells. More recently, it has been used to determine the reproductive survival of both mammary (12) and thyroid (5) epithelial cells. For both of these normal tissues, the enzymatically dispersed cells were injected into the interscapular fat pad of syngeneic recipients. The mammary cells were stimulated to proliferate by the release of pro lactin from a transplanted pituitary tumor (MiT-F4). The thyroid cells responded to the elevated levels of thyrotropin which resulted from the thyroidectomy of the recipient animals.

Parenchymal hepatocytes are normally in a nonproliferative state. However, when a portion of the liver is surgically removed, the remaining cells are rapidly stimulated to divide. The parabiotic study of Christensen and Jacobsen (2) demonstrated that, not only do the cells in the remaining lobes regenerate, but those in the liver of the intact partner also initiate replication. This study and that of Moolten and Bucher (21) indicate that the environment of the systemic circulation
after partial hepatectomy acts as a mitogen for hepatocytes. Thus, we felt that a two-thirds partial hepatectomy of the recipient animals immediately prior to cellular injection would stimulate the transplanted cells to also divide. In another report, we have shown that the LND50 value is 6 times less if the recipient animals were two-thirds hepatectomized rather than nonhepatectomized. We have also found that, when hepatocytes are transplanted into hepatectomized hosts (as described in 'Materials and Methods'), a significant fraction of the transplanted hepatocytes enter into DNA synthesis and mitosis within the first 30 hr after injection. This replicative activity decreases after 24 hr and becomes similar to the control 3 days after transplantation. Since a partial hepatectomy of the recipient animal increases the clonogenicity of the transplanted liver cells, all recipient animals were two-thirds hepatectomized immediately prior to hepatocyte injection.

When cells are irradiated, the damaged cells often can go through a number of abnormal divisions, cellular fusions, etc. before they stop dividing and ultimately die (8). At high doses of radiation, because of reduced reproductive survival, large numbers of hepatocytes must be injected in order to produce liver nodules in 100% of the injection sites. A liver nodule is usually composed of approximately 50 cells. However, if a large number of radiation-exposed cells are injected, it is difficult to determine whether a cluster of hepatocytes arose from a single viable cell or from the coalescence of many microcolonies all of which arose from abortive proliferative attempts of the radiation-sterilized parent cells. Such coalescence of microcolonies was seen when more than 100,000 hepatocytes were injected. Fig. 2b shows microcolonies which are composed of small numbers of cells with prominent radiation-induced abnormalities in the nuclei. To effectively eliminate this problem, it was found necessary to reduce the total number of hepatocytes that were injected into a site to a maximum of 100,000 cells and to use multiple injection sites when more cells were required ("Appendix"). At this cell number, a liver nodule was never observed when radiation-sterilized cells were injected. Thus, the probability of a false positive is insignificant when the total number of irradiated morphologically viable cells injected is kept below 100,000.

Since the shape of the transplantation curves is not dependent upon the dose of radiation to which the cells are exposed (Chart 2), the survival fraction can be estimated by dividing the LND50 for unexposed cells by that for exposed cells (9). The survival curve for hepatocytes generated in this manner reveals some interesting characteristics. The D0 value or mean lethal dose we have estimated for reproductive cell death is more than an order of magnitude less than that estimated for interphase death of hepatocytes (17). However, this estimated D0 value is larger than that observed for skin epithelial cells [D0 = 135 rads (31)], crypt cells of the jejunum [D0 = 97 rads (32)], bone marrow stem cells [D0 = 115 rads (28)], and mammary gland epithelial cells [D0 = 129 rads (12)] but is more comparable to that observed for thyroid epithelial cells [D0 = 200 rads (22)]. These data indicate that the D0 values for normal tissues are tissue dependent.

In a discussion on the relationship between chromosomal aberrations and cell killing, Dewey et al. (7) have stated that "only one lethal aberration (which may not always be detected and which may be accompanied sometimes by a nonlethal aberration) either is responsible for cell death, or is a manifestation of damage resulting in cell death." Coggle (4) has studied the chromosomal aberrations induced in hepatocytes by single and fractionated doses of X-rays. When he graphed the logarithm of the fraction of normal mitoses against the dose for cells which were exposed 24 hr before partial hepatectomy (i.e., while in the G0 phase), the extrapolation number was found to be 1.00 ± 0.02 (S.E.). He also found that these hepatocytes were unable to repair the damage which was responsible for the chromosomal aberrations.

In view of the association between reproductive cell death and chromosomal aberrations, the results of Coggle would predict that the reproductive survival curve of hepatocytes exposed in G0 would also have an extrapolation number close to 1. This value is similar to what we have observed, and this shows that hepatocytes irradiated while in G0 are unable to effectively accumulate sublethal damage if they are forced to proliferate within 24 hr after radiation exposure. The results of our study do not, however, address the question of whether or not hepatocytes can effectively repair X-ray-induced sublethal damage. They only indicate that in the presence of unrepaired genetic damage hepatocytes cannot undergo the sustained cellular proliferation required to produce hepatocyte nodules. The capacity of hepatocytes to repair DNA damage caused by a variety of chemicals has been demonstrated (20, 30). It has also been shown with chemical genotoxic agents (27) that sustained genetic damage inhibits replication of normal hepatocytes. For example, the continuous administration of N2-acetylaminofluorene, a chemical agent genotoxic for hepatocytes, inhibits the proliferation of hepatocytes that is normally seen during liver regeneration. The exact nature of X-ray-induced damage and the capacity of hepatocytes to repair it will be a subject of future studies.

In conclusion, we have developed an in vivo transplantation assay system which can be used to estimate the probability of reproductive survival of parenchymal hepatocytes exposed to genotoxic agents, either physical or chemical. The potential of cell replication in primary cultures of hepatocytes is low in most systems, and to our knowledge there is no in vitro clonal assay for primary cultures of parenchymal hepatocytes analogous to the clonal assays for C3H1OT½ cells or human fibroblasts (18). Thus, the system presented here is the only clonogenic assay available for parenchymal hepatocytes. We have utilized this system to determine the reproductive survival curve for parenchymal hepatocytes exposed to single doses of low-LET radiation. We are similarly determining the probability of liver cell survival after exposure to chemical carcinogens so that we can ultimately express the probability of malignant transformation on a per surviving rather than exposed cell basis.

ACKNOWLEDGMENTS

We would like to thank Dr. Mark Engler for his help with the radiation dosimetry.

APPENDIX

We want to know under what conditions the probability of liver nodule formation or "take" at one injection site for a given number of injected cells Z, P(Z), is equivalent to the probability of a take at any of n independent injection

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sites \((n = 1, 2, 3, \ldots)\) with \(1/n\) the number of cells in each site. Symbolically, we require conditions for the equality

\[
P(Z) = 1 - P(\text{No takes}) = 1 - \left[ P(\text{No takes in first site}) \times P(\text{No takes in second site}) \times \ldots \times P(\text{No takes in nth site}) \right].
\]

or equivalently

\[
1 - P(Z) = \left[ 1 - \frac{P(Z)}{n} \right]^n.
\]

From Equations A and B in the "Materials and Methods," we have

\[
1 - P(Z) = e^{-\alpha s};
\]

thus, the condition is

\[
e^{-\alpha s} = \left[ e^{-\alpha(D/n)} \right]^n
\]

or taking the natural logarithm of both sides

\[
D^2 = n(D/n)^2
\]

or

\[
1 = e^{(1 - s)}.
\]

This is true only if \(S = 1\). Thus, multiple injection sites can only be used if the hepatocyte transplantation data are described by a Poisson distribution, i.e., when

\[
P(Z) = 1 - e^{-\alpha}.
\]

REFERENCES


Fig. 1. Whole-mount view of 3 hematoxylin-stained liver nodules surrounded by white adipose connective tissue. Each nodule is composed of approximately 50 hepatocytes. × 80.
Fig. 2. a, liver nodule generated from the injection of hepatocytes not exposed to radiation. The nuclei are of uniform size. H & E, × 400. b, microcolonies generated from the injection of hepatocytes exposed to 1300 rads. Compared to the cells of a, these cells are larger and their nuclei show prominent radiation-induced changes. Note the presence of 3 nuclei of uneven size in the large cell marked by an arrow. H & E, × 400.
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