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The survival of parenchymal hepatocytes irradiated with low and high LET radiation

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Summary We have developed an *in vivo* clonogenic assay system for parenchymal hepatocytes which has enabled us to investigate the reproductive survival and repair capacity of liver cells exposed to genotoxic agents. In this report we present our results with hepatocytes irradiated with ^{60}Co and neutrons. The survival curve for parenchymal hepatocytes enzymatically dispersed 30 min after exposure to ^{60}Co has a D_0 value of 2.7 Gy and an extrapolation number insignificantly different from unity. However, when the hepatocytes were allowed to remain *in situ* for 24 h before being assayed for survival, the extrapolation number significantly increased to 2.3, whereas the D_0 value remained unchanged. Therefore, normal parenchymal hepatocytes in G_0 are able to repair potentially lethal damage (PLD) after exposure to ^{60}Co and this repair phenomenon is expressed solely as an increase in the n value. In contrast, with hepatocytes exposed to 14.3 MeV neutrons, the survival curve is exponential with a D_0 value of 1.7 Gy regardless of whether the cells remained *in situ* for 30 min or 24 h before the assessment of their reproductive survival. A comparison of the ^{60}Co and neutron survival curves, where 24 h is allowed for PLD repair to occur, demonstrates that the RBE of neutrons for hepatocytes increases with decreasing dose and equals 4.2 at 50 cGy. The radioprotective agent WR-2721 was shown to act as a dose modifying agent with a DMF of 2.1, implying that it may be of potential clinical value as a radiation hepato-protective drug.

In the adult human, the liver constitutes ~ 2.5% of the total body weight. The structural relationship between the parenchymal and vascular elements enables the liver to perform the specialized function of removing substrates absorbed by the intestines for subsequent storage, metabolism and distribution to the blood and bile. The liver is also the major site for the biotransformation of xenobiotic compounds, thereby serving as a guardian interposed between the rest of the body and the intestinal tract. However, because of this unique anatomical location, it is also a common site for development of metastases. For example, 60–70% of patients with metastatic large bowel cancer have hepatic metastases and many of these hepatic lesions exist in patients with no other evidence of distant spread. Thus, the effective treatment of this disease has the potential for saving a substantial number of lives. To accomplish this goal, much more information is needed concerning effects of radiation on the liver. As stated by Field & Michalowski (1979), "In view of the sheer size of the liver, the frequency of its metastatic cancer involvement and the vast amount of analytical work on the consequences of its irradiation, it is perhaps surprising that the organ has almost completely escaped an overall description of its long-term radiobiological properties."

One reason for this paucity of information is that without a clonal assay system for parenchymal hepatocytes one of the most important radiobiological parameters, the probability of reproductive survival, could not be estimated. In this report we describe the *in vivo* transplantation clonal assay system we have developed for parenchymal hepatocytes and present data which describe the survival characteristics for hepatocytes exposed to either ^{60}Co γ-rays (Jirtle *et al.*, 1981; Jirtle *et al.*, 1982) or 14.3 MeV neutrons (Jirtle *et al.*, 1983). The ability of liver cells to repair PLD after exposure to ^{60}Co and neutrons and the effectiveness of WR-2721 in protecting parenchymal hepatocytes from radiation damage will also be discussed.

Materials and methods

Animals

Female syngeneic rats (Fischer 344) weighing ~100 g were obtained from the Charles River Breeding Laboratories (Wilmington, MA) and were used both as hepatocyte donors and recipients in all of these experiements. They were housed in a temperature and humidity controlled room with a 12 h light-dark cycle. Autoclavable Laboratory Chow 5010 (Ralston Purnia Co., St Louis, MO) and water were provided *ad libitum*.

Irradiations

The rats were irradiated either with ^{60}Co γ radiation at a rate of 90 c Gy min^{-1} or 14.3 MeV neutrons at 20 c Gy min.$^{-1}$ For the neutron studies the hepatocyte-donor animals were irradiated at the University of Wisconsin's Gas Target Neutron Source. Monoenergetic 14.3 MeV neutrons were produced by a $^{3}H(^{2}H, n)^{4}He$(D-T) reaction. The details of the irradiation and dosimetry procedures are provided elsewhere (Jirtle *et al.*, 1981; 1983).

Studies with WR-2721

The radioprotective agent was intraperitoneally (IP) injected at a dose ranging from 50–400 mg kg^{-1} 30 min prior to ^{60}Co γ-irradiation. In all cases the hepatocytes were allowed to remain *in situ* for 24 h after radiation treatment before they were dispersed and transplanted into recipient animals to assay for their reproductive survival.

Hepatocyte preparation and transplantation

The hepatocytes were enzymatically dispersed either 30 min or 24 h after the donor animals were exposed to ionizing radiation. The *in situ* two-step collagenase perfusion technique developed by Berry & Friend (1969) was utilized for the enzymatic dispersal of the liver. This technique, as utilized by us, has been previously described in detail. Cell survival was estimated by an endpoint dilution assay system (Jirtle *et al.*, 1981). Briefly, a volume of 0.06 ml of each cellular suspension was injected interscapularly and into both axillary fat pads of each recipient animal. To stimulate proliferation of the transplanted cells, the recipient animals were subjected to a 2/3 hepatectomy 1–2 h prior to hepatocyte transplantation. Twenty days after transplantation, the recipient animals were sacrificed and the fat pads were removed and histologically prepared.

Data analyses

The percentage of the injection sites for each dilution which contained at least one liver nodule was determined with the aid of a stereo microscope. The number of hepatocytes required to produce liver nodule development in 50% of the transplantation sites (LND50) was estimated by the method of maximum likelihood as previously described (Jirtle *et al.*, 1981). The fraction of the hepatocytes which survived radiation exposure was calculated by dividing the LND50 value for unexposed cells by that obtained after exposure. The multitarget-single-hit model was utilized to describe the survival data and the parameters of the model were estimated, with their 95% confidence intervals, by the method of weighted least squares.

Results

When dispersed hepatocytes are injected into the fat pads of 2/3 hepatectomized syngeneic recipient rats, the cells proliferate to form colonies which are easily visible after the tissue has been cleared and stained with hematoxylin (Jirtle *et al.*, 1980; 1981). In Figure 1 the probability of liver nodule formation is graphed versus the average number of hepatocytes injected. From this graph the number of unexposed cells required to produce a liver nodule in 50% of the injection sites (LND50) is obtained and equals 2,100 cells (95% confidence interval, 1,600–2,700 cells). This graph also demonstrates that the degree of reproducibility of the technique is high. When the hepatocytes are exposed to ionizing radiation prior to transplantation, the LND50 is increased; and since the shape of the transplantation dose-response curve does not change (Jirtle *et al.*, 1981), the surviving fraction is estimated by simply dividing the LND50 value for unexposed cells by that for those irradiated.

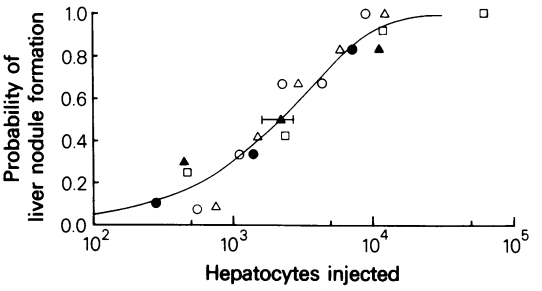

Figure 1 Probability of liver nodule formation versus the number of trypan blue negative unirradiated hepatocytes injected. The transplantation of 2,100 cells is required to produce liver nodule development in 50% of the injection sites (LND50). Each symbol is derived from 12 independent injection sites, and the different symbols represent repeat experiments. (From Jirtle *et al.* (1981) with permission of the publisher.).

The survival curve for hepatocytes enzymatically dispersed 30 min after exposure to ^{60}Co γ-rays (Figure 2) has a D_0 value of 2.7 Gy (95% confidence interval: 2.4–3.0 Gy) while the extrapolation number equals 0.9 (95% confidence interval, 0.6–1.3). If the hepatocytes were left *in situ* for 24 h before enzymatic dispersal, the resulting survival curve (Figure 2) was found to have a D_0 value which was not significantly different from 2.7 Gy. ($P > 0.1$). The two survival curves, however, were not coincident since the n value of 2.3 (95% confidence interval, 1.4–3.9) is significantly >0.9.

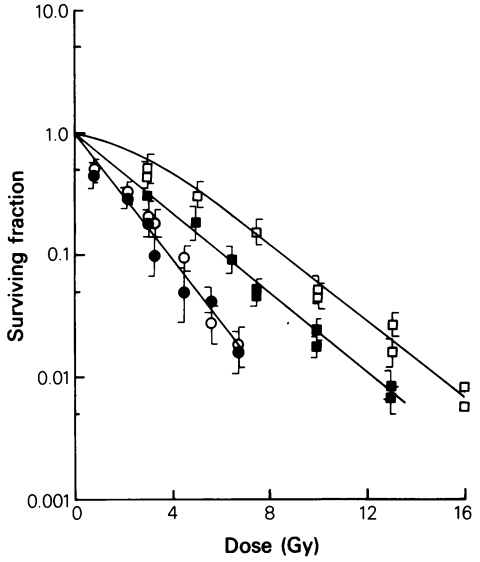

Figure 2 Surviving fraction for parenchymal hepatocytes assayed for survival 30 min (closed symbols) or 24 h (open symbols) after exposure to either ^{60}Co γ-rays (■, □) or 14.3 MeV neutrons (●, ○). The s.d. for each survival estimate is indicated by the error bars.

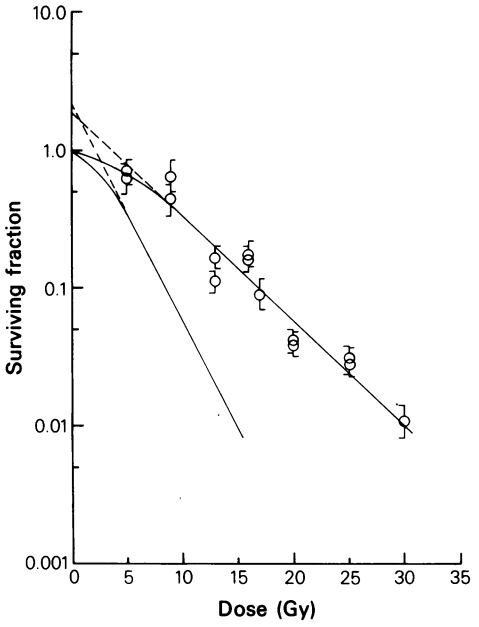

Figure 3 Surviving fraction for parenchymal hepatocytes exposed to ^{60}Co radiation 30 min after the IP injection of 400 mg kg^{-1} WR-2721. The probability of survival was estimated 24 h after radiation exposure. The survival curve for hepatocytes exposed without WR-2721 is also shown (————); the DMF is 2.1. The s.d. for each survival estimate is indicated by the error bars.

These results demonstrate that parenchymal hepatocytes do repair potentially lethal damage (PLD) after exposure to ^{60}Co radiation.

Similar studies were also performed with hepatocytes exposed to 14.3 MeV neutrons (Figure 2). In contrast to the results with low LET radiation, hepatocytes were found not to repair PLD after neutron exposure. As a consequence, the estimated surviving fractions were independent of whether the hepatocytes were enzymatically dispersed and transplanted 30 min or 24 h after the radiation exposure. The D_0 value for neutron exposed cells equals 1.7 Gy (95% a confidence interval, 1.6–1.8 Gy) and the n value is 1.0 (95% confidence interval, 0.7–1.5); thus, a simple exponential model describes the combined neutron survival data. Since neutrons are more effective in killing parenchymal hepatocytes than ^{60}Co radiation, the RBE value is greater than one and is estimated to equal 4.2 at a neutron dose of 50 c Gy.

Studies were also performed to investigate the radioprotective effect of the aminothiol, WR-2721. The results of these investigations are shown in Figure 3. In these experiments WR-2721 (400 mg kg^{-1}) was injected i.p. 30 min prior to ^{60}Co irradiation. The hepatocytes were then allowed to remain *in situ* 24 h before the cells were removed and transplanted into recipient animals. The resulting survival curve has a D_0 value equal to 5.6 Gy (95% confidence interval, 5.4–6.0 Gy) while the n value was not significantly different from that obtained without WR-2721. Therefore, under these conditions the results are consistent with WR-2721 being a dose modifying agent with a DMF equal to 2.1.

Discussion

Hepatocytes *in situ* are known to proliferate in response to a partial hepatectomy, yet their reproductive characteristics after exposure to ionizing radiation were unknown, until recently, because of the lack of a hepatocyte clonal assay. Hence, to perform the studies described herein, we first developed an *in vivo* transplantation colony forming assay system for parenchymal hepatocytes. Although we have subsequently also succeeded in stimulating hepatocytes *in vitro* to go through a limited number of mitotic divisions (Michalopoulos *et al.*, 1982), we still have not been able to obtain colony formation in culture. For this reason the *in vivo* clonal assay system still remains the only

means of assaying for hepatocyte reproductive survival.

Utilizing this system we have determined that when hepatocytes are enzymatically dispersed 30 min after radiation exposure, the resulting survival curve is adequately described by a single-hit single-target survival model with a D_0 value of 2.7 Gy; i.e., the survival data is adequately described by an exponential function. In contrast, when we allowed the irradiated liver cells to remain *in situ* for 24 h before they were assayed for their reproductive survival, the resulting survival curve had a significant shoulder (i.e., $n=2.3$) but the D_0 was unchanged. When the survival of cells is enhanced by some post-irradiation treatment, the cells are considered to have repaired potentially lethal radiation damage (PLD). As opposed to sublethal damage, the repair of PLD is demonstrated by experiments where cells are given a single dose of radiation and the post-irradiation conditions varied. Therefore, based upon this operational definition, a comparison of the 30 min with the 24 h ^{60}Co results clearly demonstrates that parenchymal hepatocytes repair PLD after exposure to low LET radiation. Previously, Gould & Clifton (1979) and Mulcahy *et al.* (1980) also observed repair of PLD in mammary gland and thyroid epithelial cells, respectively, though they referred to it as *in situ* repair since the repair phenomenon was expressed solely as an increase in the extrapolation number rather than the D_0 value, as others have observed. It remains, however, to be shown whether there is indeed a molecular difference between these two described repair phenomenon.

A comparison of the two ^{60}Co survival curves in Figure 2 also provides an interesting paradox if it is assumed that the presence of a shoulder on a single dose survival curve is attributed to the cells' ability to accumulate sublethal damage. When liver cells are assayed for survival 30 min after irradiation the resulting survival curve does not have significant shoulder; this led us initially to state that "hepatocytes irradiated while in the G_0 phase are unable to accumulate sublethal damage to an appreciable extent" (Jirtle *et al.*, 1981). In contrast, when the exposed hepatocytes are assayed 24 h after irradiation, the resulting single dose survival curve has a shoulder ($D_q=2.2$ Gy) while the D_0 value remains unchanged. Therefore, must we now conclude that hepatocytes are able to accumulate sublethal damage during radiation exposure? This contradiction is not easily reconcilable, since, as was stated by Alper (1979), "It is hardly plausible that post-irradiation treatments would have a retrospective effect on cells' capacity to accumulate sublethal damage!" One way of reconciling these conflicting conclusions is to assume that the shoulder on a survival curve simply indicates both that the repair of radiation damage occurs, and that the repair capacity is indirectly proportional to the radiation dose. Thus, if sufficient time after irradiation is not allowed for repair to occur, the survival curve will be exponential. However, if the cells are allowed time for repair to occur but the repair capacity either saturates or decreases with increasing dose, the resulting survival curve will have an initial shoulder and a terminal exponential region with a slope equal to that when the repair has not occurred. This is what we observe with hepatocytes, and thus our results are more compatible with the repair models than with the damage-accumulation models.

Parenchymal hepatocytes were also irradiated with 14.3 MeV neutrons. Again the hepatocytes were dispersed either 30 min or 24 h after irradiation in order to determine whether liver cells could also repair PLD after neutron exposure. Hepatocytes were found to be significantly more sensitive to neutrons than ^{60}Co radiation, and they were shown not to repair PLD damage after neutron exposure. However, since hepatocytes are able to repair PLD after ^{60}Co irradiation, the estimated RBE value for neutrons will depend upon both the neutron dose and the kinetics of repair of PLD after ^{60}Co exposure. When the neutron survival curve for the combined data is compared to the ^{60}Co survival curve obtained 30 min after gamma-irradiation, the RBE is simply equal to the ratio of the two D_0 values and constant at 1.6. However, when the neutron survival is compared to that obtained for ^{60}Co exposed hepatocytes after 24 h of PLD repair, the RBE values are always greater than 1.6 and increase with decreasing neutron dose to a value of 4.2 at 50 cGy.

It is of interest that the RBE estimates are longer when the degree of liver function (Geraci *et al.*, 1980), rather than reproductive survival (Jirtle *et al.*, 1983), is used as the biological endpoint. Wheldon *et al.* (1982) have recently published a provocative paper where they classify tissues as either "hierarchial" (H-type) or "flexible" (F-type). They hypothesize that in F-type tissues, protection is afforded by sub-clonogenic proliferation of the radiation sterilized cells. Therefore, they predict a dissociation of the clonogenic from the functional dose-response curves for F-type tissues. The liver provides one of the few examples of an F-type tissue where both the clonogenic survival (Jirtle *et al.*, 1982; 1983 submitted) and functional dose-response (Geraci *et al.*, 1980) curves have been obtained. A comparison of the results from these studies demonstrates that there is indeed a large dissociation between the dose-response curves for these two biological endpoints. Additionally, it is evident that the dissociation is significantly greater after exposure to low LET radiation than

subsequent to neutron irradiation. This would explain why, with F-type tissues, estimates of RBE are greater when tissue function rather than clonogenic survival is utilized as the biological endpoint.

If one used the D_0 value as an indicator of radioresistance parenchymal hepatocytes are one of the most radioresistant (Jirtle *et al.*, 1982) of the normal tissues studied. However, the shoulder width of the survival curve is a more important criterion for radioresistance when the total dose is fractionated, as in radiotherapy treatment. When the width of the shoulder, which is estimated by the D_q value, is used as an indicator of radiation sensitivity of the normal tissues studied, only bone marrow stem cells are more sensitive than parenchymal hepatocytes to low LET ionizing radiation. Thus, both the survival data for parenchymal hepatocytes and clinical experience demonstrate that the liver is an extremely radiosensitive tissue; most investigators estimate that the whole liver tolerance is 30 Gy at 2 Gy per fraction. Therefore, to treat metastatic liver cancer effectively with radiation, a method must be found to protect hepatic tissue.

The radioprotective agent WR-2721 is reputed to protect selectively many normal tissues (Yuhas *et al.*, 1980), and since it is concentrated in the liver, this drug seemed a good candidate to test for its radioprotective effectiveness. The results of these studies show WR-2721 to be efficacious in protecting hepatocytes from radiation damage; the DMF is 2.1 when 400 mg kg^{-1} is injected IP 30 min prior to radiation exposure. Hence, the D_q value is increased from 2.2–4.8 Gy. The precise role of the parenchymal hepatocyte in the aetiology of radiation hepatitis is presently not clearly understood. However, if the late onset of the loss of liver function results at least in part from parenchymal cell depletion, these results imply that WR-2721 could serve as a potent hepatoprotective agent during radiation treatment of liver metastases. Further experimental testing of this conclusion seems warranted in view of the currently poor prognosis for patients with this clinical problem.

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