A subnecrogenic dose of diethylnitrosamine is able to initiate hepatocarcinogenesis in the rat when coupled with fasting/reefeeding

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Caloric restriction causes a generalized decrease in growth rate and has been repeatedly associated with an inhibitory effect on cancer development in several systems. In contrast, exposure to complete fasting followed by refeeding is a metabolic condition associated with increased cell turnover in different organs, including the liver. The present study examines whether such condition is able to sustain the induction of initiated hepatocytes following a subnecrogenic dose of diethylnitrosamine (DENA). Male Fisher-344 rats were fasted for 4 days and 1 day after refeeding they were given a single dose of DENA (20 or 200 mg/kg body wt, i.p.). Negative and positive control groups were fed ad libitum and injected with 20 and 200 mg/kg of DENA, respectively. One week later all animals were subjected to the resistant hepatocyte model for the selection of hepatocyte nodules and they were killed 2 weeks thereafter. Results indicated the presence of gamma-glutamyltransferase (GGT) positive foci and nodules (38 ± 7/cm²) in rats regularly fed and given 200 mg/kg of DENA, while virtually no focal lesions (<1/cm²) were found in the group receiving 20 mg/kg of DENA and fed throughout the experiment. However, a significant number of GGT positive foci/nodules (14 ± 7) also developed in rats exposed to fasting and given 20 mg/kg of DENA 24 h after refeeding. No evidence of hepatocellular necrosis was found in the latter group following DENA administration. No effect of fasting was observed when rats received 200 mg/kg of DENA. It is concluded that fasting/reefeeding provides conditions which are able to sustain initiation in rat liver by a subnecrogenic dose of a carcinogen. These findings are in contrast with the commonly reported inhibitory effect of chronic food restriction on various stages of carcinogenesis, including initiation.

Introduction

The evidence indicates that diet can exert a profound effect on the evolution of neoplasia, both in animals and in humans (1–6). In particular, caloric restriction has been shown to have a delaying effect on different stages of cancer development in several experimental systems (1–9). One of the mechanisms proposed to explain these findings is that food restriction leads to a generalized decrease in the proliferative rate in several organs and, as a consequence, may affect tumor growth in a similar way (5–7). Metabolic, hormonal and immunologic alterations associated with decreased food intake have also been considered as possible contributing factors in this respect (5–11). More recent studies have suggested the possible involvement of an increased rate of cell death (apoptosis) occurring in food restricted animals as a protective mechanism against carcinogenesis (12,13).

In contrast to the large body of literature available pointing to a direct relationship between caloric restriction and cancer development, little information has been gathered on the effect of complete food withdrawal, followed by refeeding, on the evolution of neoplastic disease (13–15). The extreme condition of fasting shares some of the biological alterations induced by caloric restriction, including a decrease in the rate of cell division (13–15) and a concomitant increase in cell loss (13). However, the phase of refeeding is associated with a significant increase in the proliferative index in several organs (13–15). Given the profound influence that cell proliferation and cell loss, independently or in combination, can exert on cancer development, the overall impact of fasting/reefeeding on various phases of carcinogenic process may not be equatable in a general way to chronic food restriction per se and needs therefore to be evaluated under controlled experimental conditions.

In the present study the effect of fasting/reefeeding on the initiation step of carcinogenesis in rat liver was investigated. The results indicated that animals given a subnecrogenic dose of diethylnitrosamine (DENA*) during refeeding, following 4 days of complete food withdrawal, developed a high number of hepatocyte foci/nodules upon selection by the resistant hepatocyte (RH) protocol (16), compared to a control group (fed throughout the experiment and given the same dose of DENA) which was virtually free of focal lesions (17,18).

Materials and methods

Two-month-old male Fisher-344 rats (Charles River, Como, Italy) were used in these studies. They were acclimatized to a 12 h light/dark cycle and fed a balanced semi-synthetic diet (Piccioni, Brescia, Italy) for 1 week prior to the start of the experiments. Rats were then divided into four groups (Figure 1). Groups 1 and 2 were fasted while groups 3 and 4 were fed for a total of 4 days and then refed. Twenty-four hours thereafter animals in all groups were injected with a single dose of DENA. (Sigma Chemical Co., St Louis, MO) according to the following protocol: groups 1 and 2 received a subnecrogenic dose of 20 mg/kg body wt, i.p. (19), while groups 3 and 4 were given a necrogenic dose of 200 mg/kg body wt, i.p. (positive control). One set of animals from groups 1 and 2 were killed at 1, 2 or 3 days after DENA (three from each group per time point) while 3 rats from group 3 were killed on day 2. Blood was collected and serum enzyme analyses for alanine amino transferase (ALT) and aspartate amino transferase (AST) (GGT), gamma-glutamyltransferase; GST-P, glutathione-S-transferase.

Abbreviations: DENA, diethylnitrosamine; RH, resistant hepatocyte; ALT, alanine amino transferase; AST, aspartate amino transferase; GGT, gamma-glutamyltransferase; GST-P, glutathione-S-transferase.
DENA after fasting were indistinguishable from those found confirmed the data of GGT staining (data not shown). Histo-group 2 compared to group 3. GST-P positive lesions largely of refeeding (Table I). The mean size of GGT positive lesions was smallest in group 1, while it was significantly larger in group 3, given a necrogenic dose of DENA (200 mg/kg), as expected (17,18). Our data indicate that it is given in temporal association with a regenerative stimulus ineffective in inducing the stage of initiation in the liver unless in the group receiving the high (necrogenic) dose of the initiating carcinogen. No significant differences were found in mitotic figures (~0.5%).

Serum enzyme analysis performed 1, 2 and 3 days after the injection of 20 mg/kg of DENA showed normal levels of both ALT and AST in groups 1 and 2, indicating that such treatment is not necrogenic to hepatocytes in animals previously exposed to 4 days of fasting followed by refeeding (Table II). In contrast, elevated levels of serum ALT and AST were present in rats injected with 200 mg/kg of DENA and killed 48 h after dosing, as expected (19) (Table II). A similar pattern of results was also observed upon histologic examination: no evidence of cell death was found in the liver of rats from either groups 1 or 2 killed 1, 2 or 3 days after DENA, while extensive hepatocellular necrosis with inflammatory infiltrates was evident in animals from groups 3 and 4 killed 2 days after treatment (data not presented).

Discussion

This study provides evidence that a necrogenic dose of DENA (20 mg/kg body wt) is able to initiate rat liver carcinogenesis when administered 24 h after starting refeeding in 4-day fasted animals. No significant incidence of foci was seen in animals fed ad libitum and given the same low dose of DENA, as expected (17,18).

It is well known that a single dose of any carcinogen is ineffective in inducing the stage of initiation in the liver unless it is given in temporal association with a regenerative stimulus (17,18,21-25). Such stimulus can be provided during liver development (23,24), by additional treatments such a surgical or chemical hepatectomy (17), hormones (25) or by the carcinogen itself (necrogenic dose) (17). Our data indicate that the dietary perturbation imposed by fasting/refeeding is also able to provide the conditions sufficient to confer initiating

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**Table I.** Effect of fasting/refeeding on the growth of GGT-positive foci induced by DENA and 2-AAF

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No./cm²</th>
<th>Mean area (mm²)</th>
<th>Percentage area/total</th>
</tr>
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<tbody>
<tr>
<td>DENA 20 mg</td>
<td>0.6 ± 0.8</td>
<td>0.09 ± 0.09</td>
<td>0.23 ± 0.26</td>
</tr>
<tr>
<td>FAST + DENA 20 mg</td>
<td>14 ± 7</td>
<td>0.44 ± 0.08</td>
<td>6 ± 3</td>
</tr>
<tr>
<td>DENA 200 mg</td>
<td>38 ± 7</td>
<td>0.31 ± 0.05*</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>FAST + DENA 200 mg</td>
<td>39 ± 9</td>
<td>0.34 ± 0.09*</td>
<td>13 ± 3</td>
</tr>
</tbody>
</table>

Values expressed as means ± SD of six rats; *P < 0.001 and **P < 0.05 versus FAST + DENA 20 mg.

**Table II.** Blood plasma levels of transaminase activities

<table>
<thead>
<tr>
<th>Days after DENA</th>
<th>AST (IU/L)</th>
<th>ALT (IU/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENA 20 mg</td>
<td>77.0 ± 5.6</td>
<td>43.5 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>79.0 ± 6.3</td>
<td>51.7 ± 9.7</td>
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<tr>
<td></td>
<td>73.7 ± 3.4</td>
<td>45.5 ± 4.0</td>
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<tr>
<td>FAST + DENA 20 mg</td>
<td>71.0 ± 6.7</td>
<td>43.5 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>80.6 ± 15.0</td>
<td>59.6 ± 26.3</td>
</tr>
<tr>
<td></td>
<td>70.0 ± 4.5</td>
<td>47.0 ± 2.6</td>
</tr>
<tr>
<td>DENA 200 mg</td>
<td>648.0 ± 132.3*</td>
<td>404.0 ± 54.8*</td>
</tr>
</tbody>
</table>

Activity expressed as IU/L; AST = aspartate aminotransferase; ALT = alanine aminotransferase; values expressed as means ± SD. *P < 0.001 versus DENA 20 mg.

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**Fig. 1.** Male Fisher-344 rats were given a single dose of DENA: a subnecrogenic dose of 20 mg/kg i.p. (1,2) or a necrogenic dose of 200 mg/kg i.p. (3,4). Rats of groups 2 and 4 were fasted for 4 days following 4 days dosing, as expected (19) (Table II). A similar pattern of results was also observed upon histologic examination: no evidence of cell death was found in the liver of rats from either groups 1 or 2 killed 1, 2 or 3 days after DENA, while extensive hepatocellular necrosis with inflammatory infiltrates was evident in animals from groups 3 and 4 killed 2 days after treatment (data not presented).
potential to be first considered in this respect was whether the low dose of DENA, which is non-necrogenic under conditions of normal feeding (17,19), could cause hepatocellular necrosis when given to previously fasted and refed animals. However, no evidence of cell necrosis could be documented within 3 days after the injection of DENA in rats exposed to fasting/refeeding, using both serum enzyme analysis for ALT and AST and histological examination. In contrast, indexes of cell necrosis were highly positive in rats given the high dose of DENA and killed at 48 h post injection (Table II), as is known from the literature (19).

On the other hand, there is evidence to indicate that refeeding after fasting causes an increase in the rate of cell proliferation in several tissues, including liver (13–15,26). Such a proliferative wave may be sustained, at least in part, by previous cell loss occurring during complete food withdrawal (13). Given this context, it appears reasonable to hypothesize that the initiating potential acquired by a low, non-necrogenic dose of DENA given following fasting and refeeding, as observed in this study, may be supported by the increased cell turnover associated with such dietary condition. Direct measurements of both cell loss and cell proliferation in relation to the time of DENA administration will help in elucidating this possibility.

Irrespective of the mechanisms involved, it is important to note that the present findings are in contrast with the general observation on the protective effect of dietary restriction on various phases of carcinogenic process, including initiation (27–30). For example, caloric restriction was shown to inhibit rat mammary carcinogenesis induced by 7,12-dimethylbenz[a]anthracene when underfeeding extended from 1 week before to 1 week after a single carcinogen exposure (27). Similarly, 25% decrease in daily food intake inhibited intestinal carcinogenesis in rats induced by indirect (but not direct) acting carcinogens, possibly via an altered metabolism and a decreased binding of carcinogen to DNA (28). These types of results, when compared to those of the present study, clearly point to the existence of possible important differences between chronic caloric restriction and complete food withdrawal followed by refeeding in their respective impact on cancer development from the earliest stages. The bases for these differences need to be explored. Clearly chronic food restriction imposes a series of alterations (metabolic, hormonal and other more complex biological responses), which remain relatively constant over time. By contrast, the extreme condition of fasting induces acute responses which are then reversed upon refeeding, thus requiring metabolic and other adaptive mechanisms to continuously operate. In this respect the experimental conditions used in this study (complete food withdrawal followed by refeeding ad libitum) may be relevant to more common patterns of feeding involving significant variations in the amount of food consumed daily. Such consideration could also apply specifically to humans, particularly in certain areas of the world in which the availability of food is scarce and irregular. Also relevant to this point is our recent finding on the enhancing effect of cycles of fasting and refeeding on the growth of hepatocyte nodules selected by the RH model in rat liver (31).

In conclusion, the results of this study indicate that a low, non-necrogenic dose of DENA is able to initiate carcinogenesis in rat liver when it is administered 24 h post refeeding following 4 days of fasting. This finding is at variance with the general observation on the inhibitory effect of food restriction on carcinogenic process. More information is therefore needed before definitive conclusions can be drawn on the complex relationship between dietary habits and neoplastic disease.

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