Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 9995–9999, October 1994
Applied Biological Sciences

Food restriction eliminates preneoplastic cells through apoptosis and antagonizes carcinogenesis in rat liver

BETTINA GRASL-KRAUPP*, WILFRIED BURSCH, BRANISLAV RUTTKAY-NEDECKY, ALEXANDRA WAGNER, BERNWARD LAUER, AND ROLF SCHULTE-HERMANN

Institut für Tumorbiologie-Krebsforschung, Borschkegasse 8a A-1090 Vienna, Austria

Communicated by James A. Miller, July 8, 1994 (received for review March 21, 1994)

ABSTRACT **Restriction of dietary calories reduces cancer formation in experimental animals and probably also in humans. This effect is generally attributed to the inhibitory effect of fasting on cell proliferation. Here we studied the effect of fasting on physiological cell death through apoptosis by using rat liver as a model. (*i*) In normal liver, involution of hyperplasia by apoptosis was reinforced by food withdrawal and suppressed by feeding. Complete food withdrawal for 8 days or food reduction by 40% for 3 months eliminated 20–30% of normal liver cells through apoptosis. (*ii*) Putative preneoplastic liver foci exhibited severalfold higher rates of DNA replication and apoptosis than unaltered liver. Food restriction lowered DNA replication but increased apoptosis, which reduced the number and volume of putative preneoplastic liver foci by 85% within 3 months. Subsequent return to ad libitum feeding normalized cell replication and apoptosis but clear differences in the volume and number of putative preneoplastic liver foci persisted throughout the following 17 months. Treatment of animals after food restriction with nafenopin, a peroxisome proliferator and potent tumor promoter, produced only half as many hepatocellular adenomas and carcinomas as in animals fed unrestrictedly throughout their lifetime. This indicates that food restriction had actually eliminated a part of the initiated cells. This study demonstrates that food restriction preferentially enhances apoptosis of preneoplastic cells. This effect in combination with lowered cell replication provides protection from carcinogenesis.**

Diet appears to be the most important determinant of human cancer—i.e., about one-third of all cases are attributed to dietary factors (1). Besides carcinogenic constituents, excessive calorie intake is considered a major risk factor associated with diet (2–6). The importance of calorie intake is supported by animal experiments, in which food restriction alone greatly reduces the incidence of cancer in the liver and many other organs, while overfeeding enhances carcinogenesis (7–12).

Over the past decades, studies on the mechanisms of cancer protection by calorie restriction focused on the neuroendocrine network, overall energy expenditure, or metabolism of the main dietary constituents (8, 13). At the cellular level, the feeding/fasting state of animals was found to control cell replication in several tissues including the liver (8, 14–16).

Rodent liver offers one of the best models to study effects of environmental or endogenous variables on growth control and carcinogenesis (17, 18). In this organ, putative preneoplastic foci (PPF), which are at an intermediate stage on the pathway to cancer, can be identified by histochemical techniques, and considerable knowledge of their biological properties has accumulated (19). PPF can be induced by treatment with genotoxic carcinogens or appear "spontaneously" during aging (20). We have found (21) that PPF are more sensitive to the antiproliferative effects of food restriction than normal liver cells.

A physiological counterpart of cell replication is cell death by apoptosis. Apoptosis occurs at relatively high levels in PPF and is suppressed by tumor promoters that thereby accelerate tumor development (22). In normal liver, apoptosis is regulated by endogenous factors such as transforming growth factor β1, which inhibits DNA synthesis and triggers apoptosis (23). In the present study, we show that apoptosis in normal liver and in PPF is controlled by signals exerted by feeding or fasting.

MATERIALS AND METHODS

Animals and Treatment. SPF Wistar rats from Zentralinstitut für Versuchstierzucht (Hannover, Germany) received Altromin 1321N (Altromin, Lage, Germany) as a basal diet and were kept under standardized conditions (24), with an inverted 12-h light/12-h dark rhythm. If not stated otherwise, they were 4–6 weeks old at delivery. From 3 weeks before until the end of the dietary restriction period, all animals, including the nonrestricted controls, were housed individually and had access to food daily between the time of 0900 and 1400. This changed neither the amount of consumed food per day nor the body weight (b.w.) of the animals (data not shown). Restricted animals received daily 40% or 60% of that amount consumed by the controls the day before. When dietary restriction lasted for >24 h, vitamins, minerals, and trace elements (gratuitously supplied by Altromin) were admixed to the food to ensure intake identical to controls.

As shown before, the diurnal rhythm of DNA synthesis in the liver is synchronized to a single peak per day through rhythmic feeding (15, 22). At this peak, [^{3}H]thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq; NEN) was injected i.p. as a single dose of 0.2 mCi/kg b.w. 36 h before killing. If not stated otherwise, animals were sacrificed by decapitation under CO_2 narcosis between the times of 0800 and 0900.

Biochemical Procedures. Hepatic DNA content was determined by the diphenylamine procedure (for details, see ref. 15).

Morphology. Specimens of liver tumors measuring at least 2 mm in diameter and of liver tissue bearing no visible lesion were fixed and processed as described (24). Two serial sections of tissue, stained with hematoxylin/eosin (H&E) and for placental glutathione *S*-transferase (GST-P), served to identify PPF and liver tumors according to previous descriptions (24, 25). Since GST-P staining is insufficient for the detection of foci and tumors arising upon treatment with a peroxisome proliferator (25), specific evaluation criteria,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AB, apoptotic body; b.w., body weight; CPA, cyproterone acetate; GST-P, placental glutathione *S*-transferase; H&E, hematoxylin/eosin; NAF, nafenopin; PPF, putative preneoplastic liver foci.

*To whom reprint requests should be addressed.

which had been elaborated for these lesions in H&E-stained tissue sections (24), were applied to nafenopin (NAF)-treated livers. The measured size of the PPF served to determine the number and volume of the PPF per liver according to Saltykow (26).

Hepatocytes undergoing death by apoptosis (apoptotic bodies, ABs) were identified in H&E sections as described in detail (27). In each liver, the labeling index after autoradiography and incidence of ABs were determined by assaying at least 2000 and 4000 unaltered cells, respectively, and all cells within individual PPF. Due to small interindividual variations, we pooled data on labeling indices and ABs obtained from rats of the same treatment group and time point.

RESULTS

The Fasting/Feeding State Influences Apoptosis During Involution of Mitogen-Induced Hyperplasia. Rat liver hyperplasia was induced by repeated treatment with the hepatomitogen cyproterone acetate (CPA). As described (27), this hyperplasia is readily eliminated by apoptosis of excessive liver cells when treatment is discontinued, while retreatment with CPA prevents apoptosis. In the present experiment, in which the apoptotic activity was elevated 40 h and more after the last dose of CPA (basal rate of apoptosis ranging between 0.01 and 0.03%; data not shown), we noted that the daily feeding rhythm had a profound impact on the incidence of apoptoses (Fig. 1*A*). Apoptotic activity was high during the daily fasting period and declined significantly after the start of feeding; if food was withheld, apoptotic activity remained high. Subsequently, feeding and CPA were found to be similarly effective inhibitors of apoptosis (Fig. 1*B*). When the animals did not receive the complete diet but instead received isocaloric amounts of pure carbohydrates, fats, or proteins, each component alone was able to significantly lower apoptotic activity; thus, the energy intake *per se* rather than specific food components is effective (Fig. 1*B*). Repetition of this experiment provided similar results (data not shown).

From the present study, the maximum rate of apoptosis may be expected shortly before feeding, whereas the peak of cell replication is known to occur roughly 12 h later (15, 16, 21). Thus, the cyclic eating behavior of rats seems to result in a diurnal rhythm of cell turnover in the liver. Untreated rats tended to exhibit a similar time course of apoptosis over 24 h but the incidence was too low to display significant differences (data not shown). Apparently, the present fasting protocol had a permissive effect on the induction of apoptosis primed by preceding mitogen treatment and withdrawal.

Food Reduction Induces Apoptotic Activity in the Liver. When food was withheld from untreated young rats for 8 days, the average b.w. was reduced from 157 to 99 g. Concomitantly, liver mass decreased by ≈25% within the first 2 days and by an additional 35% more gradually thereafter (Fig. 2). A significant loss of hepatic DNA was seen after day 3. Thus, the early decline in organ mass might have been due to mobilization of glycogen, protein, and lipids, whereas the later decline probably was due to elimination of cells from the liver. In fact, from day 2 onward, there was a considerable number of ABs, with the highest levels on days 7 and 8 (Fig. 2).

When food intake of 9-month-old rats was reduced to 60% for up to 95 days, the average b.w. declined slowly by ≈30% from 417 to 297 g, and liver mass and DNA content decreased by ≈40% and 20%, respectively (Fig. 3). Immediately after return to unrestricted feeding, animals consumed much more food than controls; the mean b.w. increased to 365 g within a week. The elevated food intake provided a significant growth stimulus also for the liver as has been known for many years (28). Consequently, the liver regained its control weight within 7 days. DNA content still was slightly decreased at this time (Fig. 3) but had recovered a few weeks later (data not shown).

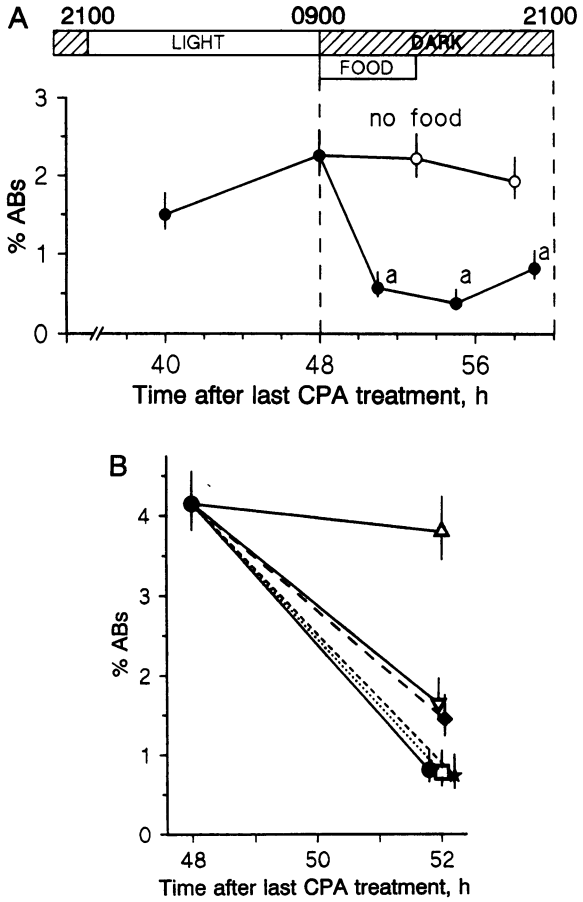

FIG. 1. Effect of fasting or of feeding dietary components on the frequency of apoptosis in regressing rat liver. Female rats were kept on rhythmic feeding between the time of 0900 and 1400 and received CPA dissolved in maize oil by gavage for 7 days as described (23, 27). (*A*) Forty-eight hours after the last application of CPA, rats were either given (○) or denied (●) access to food. Animals were sacrificed between 40 and 59 h after CPA treatment. (*B*) Forty-eight hours after the last CPA dose, food was withheld from rats, which then received by gavage one dose of CPA (130 mg/kg) suspended in water with 0.09% mirj 52 (Serva) (▽), solvent only (△), or isocaloric amounts (628 kJ/kg b.w.) of fat (18 ml of pure maize oil per kg b.w.) (●), of carbohydrates (37 g of dextrin in 50 ml of tap water per kg b.w.) (◆), of proteins (21.3 g of bovine serum albumin and 15.7 g of casein hydrolysate in 50 ml of tap water per kg b.w.) (*), or of all three dietary components together (28 g of dextrin/7 g of casein hydrolysate in 50 ml of tap water per kg b.w./0.7 ml of maize oil per kg b.w.) (□). The experiment was terminated 4 h later. Incidences of ABs with 95% confidence limits are given. In *A* significant differences from controls are indicated by an a. In *B* the solvent control was significantly different ($P < 0.01$) from groups receiving CPA, complete diet, or the three dietary components alone (pairwise testing by Wilcoxon's test).

During the 95-day fasting period, DNA synthesis of hepatocytes almost completely ceased while apoptotic activity was on a low marginally increased level, which was obviously sufficient to eliminate 20% of hepatic DNA in the virtual absence of the formation of new cells. After 7 days of unrestricted feeding, DNA replication and frequency of apoptosis had normalized to the prefasting level (Fig. 4*A*).

Restricted Feeding Induces Apoptosis and Suppresses DNA Synthesis in PPF. PPF appeared spontaneously in the livers of untreated 9-month-old rats. As in carcinogen-induced PPF (22), both DNA replication and apoptotic activity were 5- to 10-fold higher than in the surrounding unaltered liver during

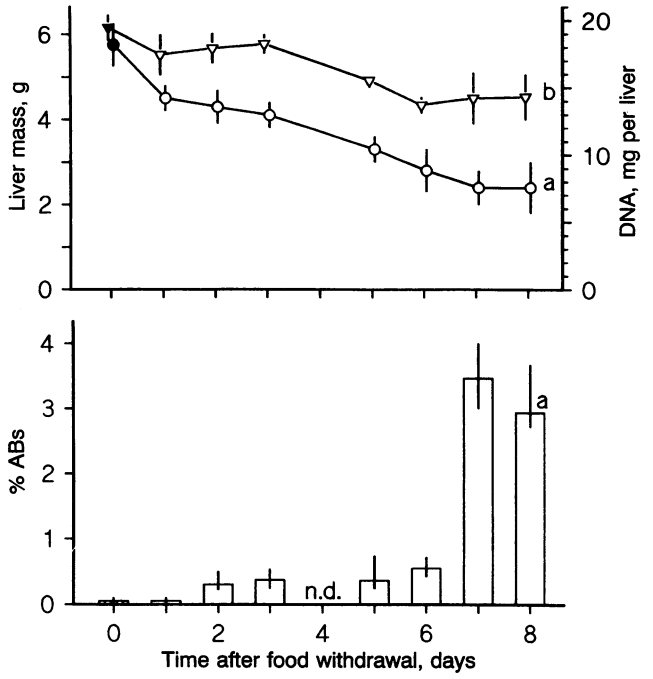

FIG. 2. Effect of complete food withdrawal for 1–8 days on liver DNA content and apoptosis. Female rats were fed ad libitum (no feeding rhythm) before the start of experimentation. The mean and SD of organ mass (circles) and DNA content (inverted triangles) and incidence of ABs with 95% confidence limits are given. There were three to six animals per group and time point. Solid symbols, ad libitum feeding; open symbols, no food. Correlations of values with duration of treatment by Spearman's test are as follows: a, $P < 0.001$; b, $P < 0.01$. n.d., Not determined.

ad libitum feeding (Fig. 4*A*). Dietary restriction to 60% of control levels strikingly depressed replicative DNA synthesis in PPF but doubled the frequency of ABs. Return to ad libitum feeding reversed the relation between DNA replication and ABs in PPF to the prefasting level.

In a further study, PPF were induced by the genotoxic carcinogen *N*-nitrosomorpholine. Probably due to the young age of the animals and the pretreatment with *N*-nitrosomorpholine, cell turnover in these PPF and in normal liver was

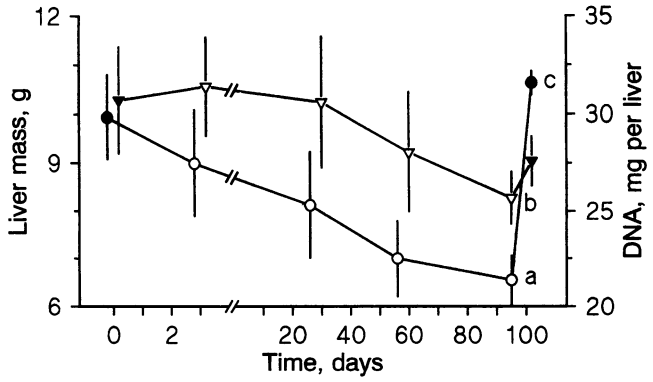

FIG. 3. Effect of a 40% reduction in food for 95 days and subsequent refeeding. Nine-month-old male rats were pair-fed 60% of the daily food ration of controls for 1, 3, 11, 28, 59, and 95 days and, subsequently, fed ad libitum for 7 days. Circles, liver mass; inverted triangles, DNA; solid symbols, ad libitum feeding; open symbols, restricted feeding. Data are the mean and SD from four or five animals. Correlations of values with duration of food restriction were calculated by Spearman's test (a, $P < 0.001$; b, $P < 0.05$). Statistical comparison at day 95 of the fasting period vs. day 7 of refeeding was done by Wilcoxon's test (c, $P < 0.01$).

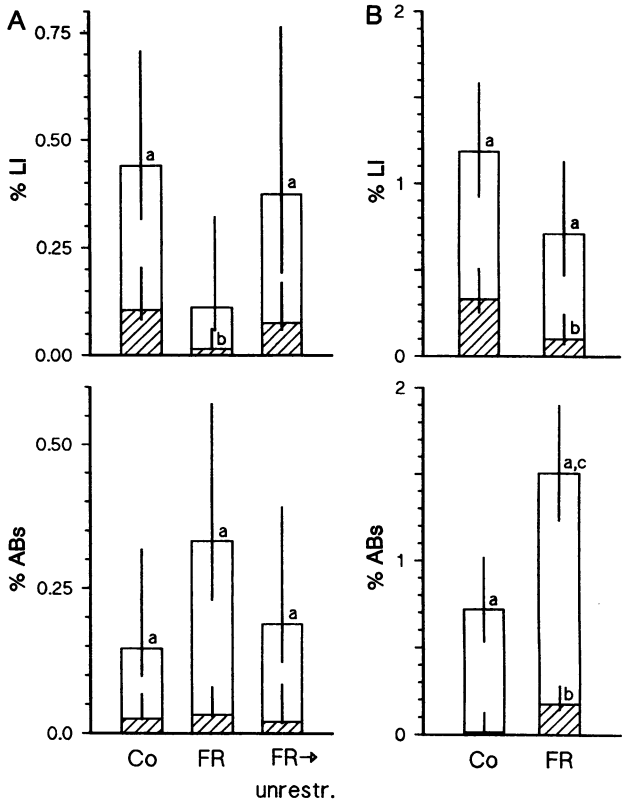

FIG. 4. Effect of food restriction on DNA synthesis and apoptosis in normal liver and PPF. (*A*) Rats described in Fig. 3 were studied. (*B*) Ten male rats, 6 weeks old, received a single dose of *N*-nitrosomorpholine by stomach tube (250 mg/kg b.w. in water; details in ref. 22). Fifteen weeks later half of the *N*-nitrosomorpholine-treated animals were pair-fed at 40% of the control level for 4 days. The frequency of ABs and labeling index (LI) in PPF, identified by H&E and GST-P staining (total column), and in the surrounding unaltered liver (hatched part of the column) are given as incidences with 95% confidence limits. (*A*) Data obtained at the various time points were pooled. At least 5000 cells within PPF were counted per treatment group. Statistical significance ($P < 0.05$): a, PPF vs. surrounding normal liver; b, normal liver of unrestricted vs. restricted animals; c, PPF of unrestricted vs. restricted animals. Bars: Co, fed ad libitum (control); FR, food restriction.

rather high (Fig. 4*B*; note different scales of ordinates in Fig. 4 *A* and *B*). Dietary restriction to 40% of control levels for 4 days suppressed DNA replication by ≈40% and significantly induced apoptosis ≈2-fold, which confirms the effects of fasting in the "spontaneous" PPF described above.

Remarkably, all these changes in the PPF were qualitatively similar to those occurring in normal liver but were significantly more pronounced. Thus, PPF appear to be preferentially sensitive to signals modulating DNA replication and apoptosis in response to feeding or fasting.

Persistent Effects of Fasting on Early and Late Stages of Hepatocarcinogenesis. After food restriction for 95 days (Figs. 3 and 4*A*), the "spontaneous" PPF, as a consequence of the shift from cell generation toward elimination, showed a dramatic decline of their mean volume and number, so that their total volume was reduced to ≈15% (Fig. 5 *A* and *B*). Subsequent refeeding provided a considerable growth stimulus not only for the normal liver (Fig. 3) but also for the PPF so that within 1 week the total number and volume of the PPF increased from 15 to ≈65% of the controls (Fig. 5 *A* and *B*). Thereafter, the difference of ≈35% persisted during the following 17 months of ad libitum feeding (data not shown) and was not reduced by additional treatment with the tumor promoter NAF (Fig. 5 *A* and *B*).

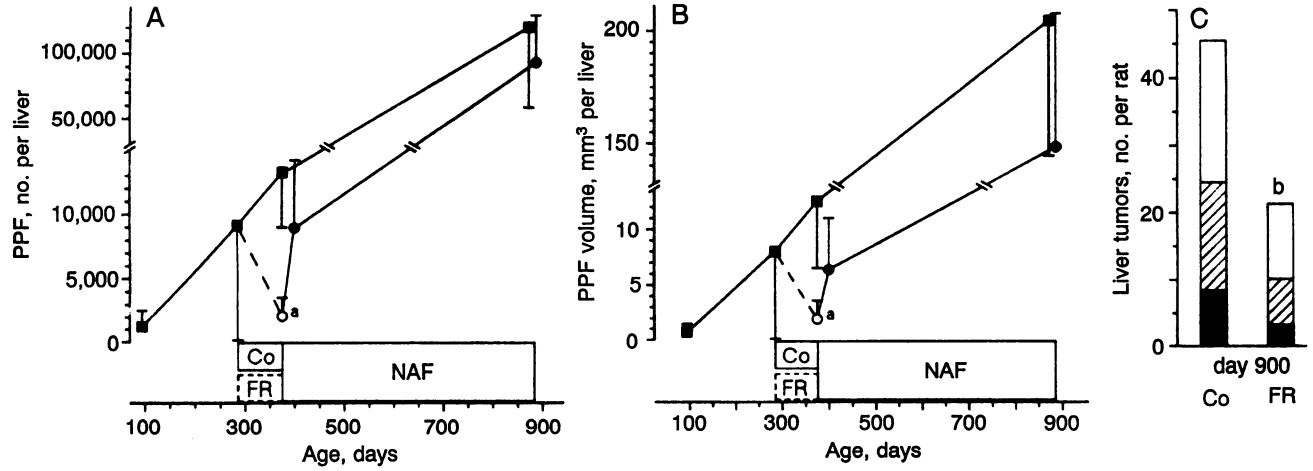

FIG. 5. Effect of food restriction for 95 days on number and volume of spontaneously occurring PPF and on the formation of liver tumors by NAF treatment. Animals and feeding schedule are as in Figs. 3 and 4A and are indicated by bars above abscissas. (A and B) Numbers and total volume of the PPF as identified by H&E and GST-P staining are given as the mean with SD. Five to 10 rats were evaluated per time point at days 94, 285, and 380 of age (■), after food restriction for 95 days at day 380 (○), after an additional 7 days of unrestricted feeding (●), and after a further 17 months of an ad libitum diet containing NAF (daily NAF dose, 100 mg/kg b.w. as described in ref. 24) (● and ■). (C) Liver tumors after NAF treatment in rats formerly fed a restricted diet (FR; $n = 13$) and in controls fed an unrestricted diet (Co; $n = 19$). Total column, number of macroscopically detectable lesions per liver; hatched/solid parts, hepatocellular adenoma/carcinoma; open parts, macroscopical lesions <2 mm in diameter, no histology. Data are the mean; the SD is not shown. Statistics by Wilcoxon's test for Co vs. FR are as follows: a, $P < 0.005$; b, $P < 0.01$ (valid for macroscopical lesions, adenomas, and carcinomas).

To estimate the number of initiated (i.e., promotable) cells in the liver, we administered NAF for a period of 17 months. This agent has been shown to be a potent tumor promoter with little if any initiating activity (24, 29). In the present experiment, the total yield of hepatocellular adenomas and carcinomas in the restricted rats was ≈50% of that in animals fed unrestrictedly throughout their lifetime (Fig. 5C). This indicates that food reduction had actually eliminated initiated cells.

DISCUSSION

The present study demonstrates that reduction of the daily calorie intake induces apoptosis in normal liver and preferentially in PPF. This conclusion is based on three independent lines of evidence: (*i*) direct histological observation of enhanced apoptotic activity, (*ii*) reduction of total liver DNA and in particular of the number and volume of PPF, and (*iii*) reduced number of tumors after promoter treatment. Thus, a period of restricted feeding diminishes the cell population serving as target for tumor promoters and dramatically lowers the risk of cancer formation. Implications of these findings are as follows:

(*i*) Homeostasis of cell number is determined by the delicate balance between birth and death of the cells. Previous (15, 21) and the present studies suggest that the amount of consumed food, independent of its composition, largely determines which of the two processes is predominant. While fasting stops the generation of new cells and favors apoptosis in existing cells, feeding exerts the opposite effect.

(*ii*) PPF and normal liver showed qualitatively similar changes in replication and apoptosis rates during feeding or fasting. Nevertheless, PPF exhibited preferential growth or regression. Thus, food restriction for 95 days eliminated 15–20% of normal hepatocytes but 85% of preneoplastic cells. This remarkable observation can be explained by the quantitative difference in birth and death rates between normal liver and PPF. Our experimental data show that the basal rate of apoptosis was 5- to 10-fold higher in PPF than in normal liver; doubling of this rate will result in a 5- to 10-fold greater loss of cells from PPF than from normal liver. Thus, the elevated cell turnover in PPF appears to be a critical property of the preneoplastic state, because shifts in the balance between cell replication and apoptosis have a much greater impact on cell number in preneoplastic tissue than in unaltered tissue.

(*iii*) In terms of the multistage concept of carcinogenesis, it can be concluded from the present study that restricted feeding has an antipromoting effect on carcinogenesis in rat liver as the mean and total volume of PPF were dramatically decreased. However, the decrease of the mean size of individual PPF vanished soon after return to ad libitum feeding, which suggests that the antipromoting effect was readily reversible.

(*iv*) Dietary restriction probably eliminated a considerable number of initiated cell clones, as suggested by the observed decrease in numbers of foci and tumors. The steep decline in the mean volume of PPF induced by fasting might have put many PPF at risk for extinction. In fact, mathematical modeling shows that the probability of extinction of a preneoplastic clone is inversely proportional to its size (30). Therefore, fasting, by its effect on the balance between cell birth and death, may counteract initiation, at least partially. In conclusion, the present study provides a mechanistic explanation for the repeatedly observed antiinitiating and antipromoting effect of food reduction (7, 8, 11, 31, 32).

(*v*) An important question is whether periods of dietary restriction may also have cancer-preventing effects in humans. According to epidemiological studies high consumption of energy in form of fat is directly correlated with the mortality due to cancer of breast, colon, or prostate in many countries throughout the world (1, 4–6). Although it is difficult to differentiate the effect of fat from that of energy intake, experimental data suggest that reduction of energy intake is an effective way to reduce cancer risks (9, 11, 33–36). The prevention of cancer by food reduction in many different species supports the probability that fasting is of benefit also for humans (7–12). The present results obtained with spontaneously occurring PPF may be of particular relevance since events similar to spontaneous initiation in rat may be predominant in the etiology of human cancer. Further support for the applicability of the present findings stems from observations on apoptosis in various human tissues

including those apparently at a higher cancer risk due to excessive calorie intake (37–39).

In conclusion, if the present findings apply to human organs, fasting may be a simple method to eliminate dangerous preneoplastic cells from the organism and may provide great benefit for cancer prevention.

The excellent technical assistance of B. Bukowska and H. Koudelka is gratefully acknowledged. For the gratuitous supply of essential nutrients, we thank Altromin, Lage, Germany. This study was supported by the "Anton Dreher Gedächtnisschenkung für medizinische Forschung."

1. Doll, R. & Peto, R. (1981) *J. Natl. Cancer Inst.* **66,** 1191–1309.
2. Miller, J. A. & Miller, E. C. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35,** 1316–1321.
3. Ames, B. N., Magaw, R. & Gold, L. S. (1987) *Science* **236,** 271–280.
4. Willett, W. C., Stampfer, M. J., Golditz, G. A., Rosner, B. A. & Speizer, F. E. (1990) *N. Engl. J. Med.* **323,** 1664–1672.
5. Prentice, R. L. & Sheppard, L. (1990) *Cancer Causes Control* **1,** 81–97.
6. Henderson, M. M. (1992) *Cancer Res. (Suppl.)* **52,** 2030s–2034s.
7. Tannenbaum, A. & Silverstone, H. (1953) *Adv. Cancer Res.* **1,** 451–501.
8. Weindruch, R. & Walford, R. L. (1988) *The Retardation of Aging and Disease by Dietary Restriction* (Thomas, Springfield, IL).
9. Albanes, D. (1987) *Cancer Res.* **47,** 1987–1992.
10. Birt, D. F., Salmasi, S. & Pour, P. M. (1981) *J. Natl. Cancer Inst.* **67,** 1327–1332.
11. Kritchevsky, D., Weber, M. M. & Klurfeld, D. M. (1984) *Cancer Res.* **44,** 3174–3177.
12. Pollard, M., Luckert, P. H. & Snyder, D. (1989) *Cancer* **64,** 686–690.
13. Youngman, L. D., Park, J.-Y. K. & Ames, B. N. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9112–9116.
14. Lok, E., Scott, F. W., Mongeau, R., Nera, E. A., Malcolm, S. & Clayson, D. B. (1990) *Cancer Lett.* **51,** 67–73.
15. Schulte-Hermann, R. (1977) *Cancer Res.* **37,** 166–171.
16. Barbiroli, B. & Potter, R. V. (1971) *Science* **172,** 738–741.
17. Michalopoulos, G. K. (1992) *Lab. Invest.* **67,** 413–415.
18. Lombardi, B. (1988) *Chemical Carcinogenesis* (Plenum, New York), pp. 563–581.
19. Pitot, H. C. (1990) *Annu. Rev. Pharmacol. Toxicol.* **30,** 465–500.
20. Schulte-Hermann, R., Timmermann-Trosiener, I. & Schuppler, J. (1983) *Cancer Res.* **43,** 839–844.
21. Schulte-Hermann, R., Schuppler, J., Timmermann-Trosiener, I., Ohde, G., Bursch, W. & Berger, H. (1983) *Environ. Health Perspect.* **50,** 185–194.
22. Schulte-Hermann, R., Timmermann-Trosiener, I., Barthel, G. & Bursch, W. (1990) *Cancer Res.* **50,** 5127–5135.
23. Oberhammer, F. A., Pavelka, M., Sharma, S., Tiefenbacher, R., Purchio, A. F., Bursch, W. & Schulte-Hermann, R. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5408–5412.
24. Kraupp-Grasl, B., Huber, W., Taper, H. & Schulte-Hermann, R. (1991) *Cancer Res.* **51,** 666–671.
25. Grasl-Kraupp, B., Waldhör, T., Huber, W. & Schulte-Hermann, R. (1993) *Carcinogenesis* **14,** 2407–2412.
26. Saltykow, S. A. (1967) *Proceedings of the Second International Congress for Stereology* (Springer, Berlin), pp. 163–173.
27. Bursch, W., Taper, H. S., Lauer, B. & Schulte-Hermann, R. (1985) *Virchows Arch. B* **50,** 153–166.
28. Leduc, E. H. (1949) *Am. J. Anat.* **84,** 397–400.
29. Préat, V., Lans, M., de Gerlache, J., Taper, H. & Roberfroid, M. (1986) *Jpn. J. Cancer Res.* **77,** 629–638.
30. Moolgavkar, S. H., Luebeck, E. G., de Gunst, M., Port, R. E. & Schwarz, M. (1990) *Carcinogenesis* **11,** 1271–1278.
31. Tannenbaum, A. (1944) *Cancer Res.* **4,** 673–677.
32. Sylvester, P. W., Aylsworth, C. F., Van Vugt, D. A. & Meites, J. (1982) *Cancer Res.* **42,** 4943–4947.
33. Klurfeld, D. M., Weber, M. M. & Kritchevsky, D. (1987) *Cancer Res.* **47,** 2759–2762.
34. Freedman, L. S., Clifford, C. & Messina, M. (1990) *Cancer Res.* **50,** 5710–5719.
35. Boissonneault, G. A., Elson, C. E. & Pariza, M. W. (1986) *J. Natl. Cancer Inst.* **76,** 335–338.
36. Welsch, C. W., House, J. L., Herr, B. L., Eliasberg, S. J. & Welsch, M. A. (1990) *J. Natl. Cancer Inst.* **82,** 1615–1620.
37. Ferguson, D. J. P. & Anderson, T. J. (1981) *Virchows Arch. A* **393,** 193–203.
38. Hague, A., Manning, A. M., Hanlon, K. A., Huschtscha, L. I., Hart, D. & Paraskeva, C. (1993) *Int. J. Cancer* **55,** 498–505.
39. Kyprianou, N., English, H. F. & Isaacs, J. T. (1990) *Cancer Res.* **50,** 3748–3753.