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Inhibiting effect of a hepatoma extract on the mitotic rate of regenerating liver*

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Summary. Aqueous tumor extracts were prepared by the homogenization of a fast-growing, undifferentiated, transplantable malignant murine hepatoma in distilled water. After centrifugation, an aliquot of 0.01 ml of the supernatant g body weight was injected intraperitoneally into partially hepatectomized mice. Control animals were injected with saline. Groups of mice were killed at various times in relation to the hepatectomy. Four h before killing the animals were given Colcemid (1 μg/g body weight). The number of Colcemid-arrested mitoses in the hepatocytes and in the littoral cells, respectively, were counted in 140 microscopic fields. The extract significantly inhibited the mitotic rate in hepatocytes when the injection was given between 22 h before, and up to 26 h after hepatectomy. In the littoral cells, a slight initial stimulation was followed by a slight but significant inhibition which occurred when the injection was given at hepatectomy or until 18 h after hepatectomy. The effect was not modified by exposing the extracts to temperatures of 47° C for 30 min or 22° C for 24 h, but 10 min of boiling destroyed their inhibitory effect. Lyophilization and storing at −18° C for up to 4 weeks did not modify the effect. The mitosis-inhibiting effect was also measurable when the extract was injected subcutaneously. There was an almost linear dose-response curve. The results are discussed in relation to circadian rhythms, the pattern of liver cell proliferation after hepatectomy, and recent similar reports from the literature. The conclusion is drawn that extracts of a hepatoma contain one or more growth-inhibitory factors significantly active on regenerating liver cells, and less significantly on littoral cells.

Key words: Chalone – Colcemid – Hepatoma extract – Inhibiting effects – Mitotic rates – Regenerating liver

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Introduction

The effects of endogenous growth factors on the proliferation, differentiation and maturation of cell populations have been a focus of attention in recent years (Iversen 1981, 1985). Convincing results have been obtained in cell culture with growth-stimulating factors derived from nerve, epidermis and platelets. This interesting field of research received a fresh impetus when it was shown that some of the recently discovered oncogenes either code for, or are very similar to, growth factors or cell-surface receptors for growth factors (for reviews on growth factors, see Baserga 1981; Sporn and Roberts 1985; Weinberg 1985).

Tissue and cell extracts with growth-inhibitory effects have been demonstrated in many organs and in cultured cell lines (Iversen 1981) and these factors were termed chalones by Bullough (1962). Theories of growth control have been proposed on the basis of such endogenous inhibitory signals (Weiss and Kavanau 1957) but attempts to purify these substances have not yet been as successful as those for the stimulatory growth factors.

In the liver, the situation seems extremely complex (Echave Llanos 1963, 1967a). Several studies have indicated that hepatic growth-inhibitory endogenous substances are produced. In the normal liver the rate of cellular proliferation is extremely low, but after partial hepatectomy pronounced waves of increased cell proliferation occur during the regenerative period. Liver growth control in vivo can therefore be studied after partial hepatectomy. In order to study putative growth-inhibitory substances from the liver or from malignant tumors, it is essential to have a thorough knowledge of circadian rhythms in the liver and of the pattern of regeneration after partial hepatectomy.

In previous papers we have analysed the physiological circadian rhythms of growth in the intact liver (Echave Llanos (1967b; Echave Llanos et al. 1971; Surur et al. 1984), and the circadian rhythm-related waves of proliferation in regenerating liver have been studied by Bade et al. 1966. On the basis of this information, we determined the time of the day suitable for partial hepatectomy (Souto and Echave Llanos 1985), and the correct post-hepatectomy time period for measuring the maximum inhibitory effect to tissue extracts (Echave Llanos 1967b). These are the times most suitable for partial hepatectomy, and when an injection of an extract produces maximum inhibition of the first post-hepatectomy regeneratory peak in the mitotic rate.

In the present paper we describe the inhibiting effect of an extract made from a malignant hepatoma on the mitotic rate in regenerating liver, combined with a detailed time pattern of the inhibitory effect of this extract. We also report on some of the properties of the active principle found in the tumor extract, a principle which is not yet purified or chemically characterized, but which evidently acts in a reproducible manner.

Materials and methods

The tumor. Our ES2 heptatoma is derived from the second spontaneous tumor obtained from a C3HS strain of mice. It was taken from a 625-day-old female mouse and transplanted

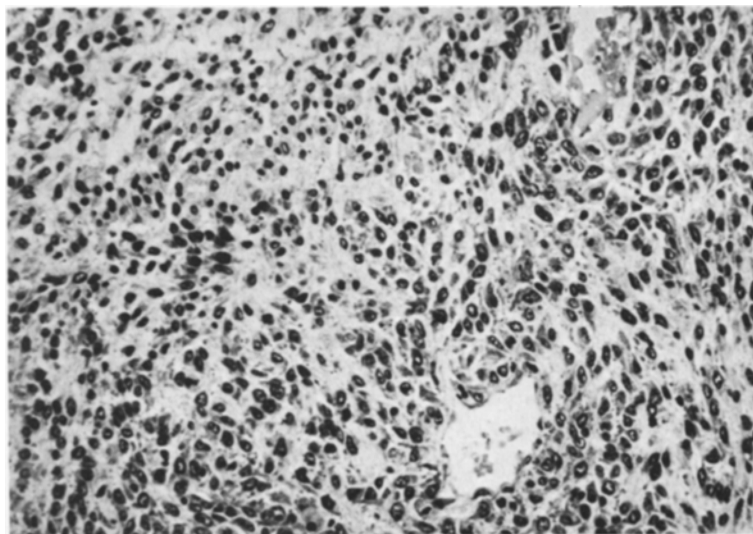

Fig. 1. Photomicrograph of an ES2 hepatoma, initial magnification 250 ×

into adult C3HS females, where it is easy to maintain. Microscopically, the tumor is an undifferentiated hepatoma with small cells exhibiting hyperchromatic nuclei and basophilic cytoplasm (Fig. 1). Local invasion is usual, but metastases are exceptional. The tumor takes in 99% of subcutaneously transplanted hosts, becoming palpable 5 days after transplantation and reaching a mean diameter of 2 cm in 15 days. It leads to the death of 50% of the animals in 30 days. The experiments reported here have been performed with transplantation generations from 80 to 100.

The extracts. Crude extracts of the tumor were prepared by pooling 3–4 tumors from different hosts. The tumors were removed surgically, and areas without necrosis or haemorrhages were selected and homogenized in a Elvehjem-Potter tissue grinder in an ice bath. Distilled water at 4° C was added in proportion 9:1. Ten strokes of the embolus were applied, and the homogenate was centrifuged at 2,000 RPM at 4° C for 20 min. The supernatant was immediately injected intraperitoneally into recipients, or treated in various ways before injection, as described below. All manipulations were performed under aseptic conditions and all the instruments were cooled before use.

The extract was administered in a dose of 0.01 ml of supernatant/g body weight of the recipient. The controls received saline only.

The recipients. A total of 382 C3HS male mice, aged 90 days, were used as recipients. All had been weighed weekly since weaning and animals with abnormal weight curves were eliminated. Mice from the same litter were randomly selected for control and experimental groups. During the last 3 weeks before partial hepatectomy the animals were standardized for periodicity analysis, i.e. caged singly in a room specially designed for this purpose, at a temperature of $22 \pm 1°$ C, with a controlled light/darkness rhythm (06.00/18.00 hours). They were given water and food *ad libitum*. One person only, and always the same one, was allowed to enter the animal room once a day with water and food.

The hepatectomy, and injections of extract, saline, and Colcemid. A partial hepatectomy comprising 70% of the liver was always made at 14.00 h. Either extracts or saline were given as a single intraperitoneal injection at various times relative to partial hepatectomy, i.e. 46 and 22 h *before* hepatectomy, *at* hepatectomy, and 10, 18, 22, 26, 32, 34, and 41 h *after* hepatectomy. All the animals were killed 46 h after hepatectomy, i.e. at noon two days after surgery. Consequently, the extract injections were given at various times of the day. Colcemid 1.0 μg/g of

body weight in 0.01 ml of distilled water was injected intraperitoneally into all the animals 4 h before killing.

Determination of mitotic activity. The animals were killed by decapitation and exsanguination 4 h after the Colcemid injection. The liver remnant was removed and fixed in 4% buffered formalin. Sections were embedded in celloidin-paraffin, cut at 5 μm, and stained with hematoxylin and eosin. The mitotic indices of hepatocytes and the sinusoidal littoral cells were determined in histological slides counting the relative number of Colcemid-arrested metaphases in relation to the number of cells in 140 microscopic fields at a magnification of 1,000×. The numbers of hepatocytes and littoral cells were determined in 14 fields) to establish the number of cells per field. The mitotic index was defined as the relative number of Colcemid-arrested metaphases per 1,000 nuclei. Since it represents 4 h accumulation of Colcemid-arrested mitoses, it is also an index of the rate of cell entrance into mitosis. Differences were analysed by Students *t* test.

Assessing properties of the extract. We learned from our study that the best period for administering the extract to obtain a maximum growth-inhibitory effect at the first mitotic peak was 26 h after partial hepatectomy, i.e. at 16.00 hour the day after partial hepatectomy performed at 14.00 hour. To measure some of the properties of the inhibitory principle in the extract, we therefore performed the partial hepatectomy at 14.00 hour and 26 h later we injected extracts that had been treated in various ways (see below), before killing the animals 48 h after partial hepatectomy.

The extracts were prepared and administered as follows: 1) Fresh extract was injected intraperitoneally into intact mice. 2) Fresh extract was injected intraperitoneally into partially hepatectomized mice. 3) Fresh extract was heated for 30 min at 47° C and injected intraperitoneally into partially hepatectomized mice. 4) Fresh extract was treated for 10 min at 100° C and injected intraperitoneally into partially hepatectomized mice. 5) Fresh extract was treated for 25 h at 22° C and injected intraperitoneally into partially hepatectomized mice. 6) Fresh extract was lyophilized and stored at −18° C for 1 week and injected intraperitoneally into partially hepatectomized mice. 7) Fresh extract was lyophilized and stored at −18° C for 4 weeks and injected intraperitoneally into partially hepatectomized mice. 8) Fresh extract was injected subcutaneously into partially hepatectomized mice. 9) Fresh extract was injected intraperitoneally in various doses (1.0, 0.5, and 0.25 mg) into partially hepatectomized mice.

In all these experiments control groups injected intraperitoneally with saline were also studied.

Results

The results are given in Tables 1 and 2, and in Figs. 2–4. Figure 2 shows the hepatocyte mitotic rates at the first peak after hepatectomy, after saline injection, and after extract injection. Figure 3 shows the same values for the littoral cells. In Fig. 4 both hepatocyte and littoral cell mitotic rates are shown as ratios of the values seen in saline-injected animals set at 1.00.

The injection led to significant inhibition of the mitotic rate in hepatocytes when the extract was given during the time period from 22 h before to 22–26 h after partial hepatectomy. Maximum inhibition occurred when the extract was injected at the time of partial hepatectomy. In the littoral cells, extract injection 22 h before partial hepatectomy gave rise to significant stimulation of mitotic activity, whereas injection 10 and 18 h after partial hepatectomy gave rise to significant inhibition.

Table 2 shows the effects of the various treatments of the extract. The low mitotic activity of hepatocytes in intact adult liver was not altered by the extract, but it significantly inhibited the mitotic activity in rapidly

Table 1. Action of the time of injection on the inhibiting effect of ES2 fast growing indifferentiated hepatoma crude extracts on the mitotic activity of regenerating liver hepatocytes and sinusoid littoral cells populations, controlled at the first peak time: 14:00/48 (TD/TR: Time of day/Time of regeneration). % I: Percent of inhibition. % S: Percent of stimulation. $p<$: Statistical significance (Student's "t test"). $\bar{x}+$SE (n): Mean ±1 Standard error (n; sample size)

Injection Time TD/TR	Hepatocytes mitotic index 12:00 /46 h						Littoral cells mitotic index 12:00/46 h						
	Saline		Extract				Saline		Extract				
	$\bar{x}$ ±SE	(n)	$\bar{x}$ ±SE	(n)	% I	$p<$	$\bar{x}$ ±SE	(n)	$\bar{x}$ ±SE	(n)	% I	% S	$p<$
16:00/−46	51.3± 8.0	(6)	54.1±4.4	(6)	–	–	10.8±1.0	(6)	8.4±1.3	(6)	22	–	–
16:00/−22	82.8±22.2	(6)	8.1±1.7	(6)	90	0.01	8.5±1.3	(6)	32.3±2.4	(6)	–	380	0.01
14:00/ 00	76.5±21.1	(5)	1.5±0.5	(5)	98	0.02	20.0±6.1	(5)	16.1±0.9	(5)	20	–	–
00:00/ 10	40.9± 8.7	(6)	1.3±0.2	(6)	97	0.01	25.5±4.7	(6)	13.4±2.1	(6)	47	–	0.05
08:00/ 18	65.9±14.4	(6)	2.4±1.0	(6)	96	0.01	28.7±3.1	(6)	14.3±3.7	(6)	50	–	0.02
12:00/ 22	68.5± 8.6	(6)	5.5±2.2	(6)	92	0.001	17.3±3.8	(6)	13.8±2.0	(6)	20	–	–
16:00/ 26	83.6±10.2	(6)	14.7±4.1	(6)	82	0.001	6.4±0.8	(6)	3.4±1.3	(6)	47	–	–
22:00/ 32	52.1± 2.4	(5)	28.8±4.9	(5)	45	0.001	8.9±2.1	(5)	5.4±1.0	(5)	39	–	–
00:00/ 34	57.3± 8.0	(6)	40.1±9.5	(6)	30	–	3.6±1.3	(6)	4.8±1.6	(6)	–	25	–
07:00/ 41	74.8±10.7	(10)	43.5±4.0	(6)	48	0.05	13.8±3.3	(10)	11.2±3.7	(6)	19	–	–

Table 2. Action of fresh extract of ES2 indifferentiated fast growing hepatoma on mitotic acitivity (Mitotic index: Colchicine metaphases/1000 nuclei) of intact and regenerating liver. Action of different treatments on the inhibiting effect observed on regenerating liver. Dose/Response curve

Treatment of the extract	Hepatocytes					Sinusoid littoral cells				
	Saline		Extract		$p<$	Saline		Extract		$p<$
	$\bar{x}$ ±SE	(n)	$\bar{x}$ ±SE	(n)		$\bar{x}$ ±SE	(n)	$\bar{x}$ ±SE	(n)	
Intact liver (12:00 h)										
1: Fresh extract (FE)	0.2± 0.2	(6)	0.0±0.0	(6)	–	1.3±0.3	(6)	2.7±0.6	(6)	–
Regenerating liver (12:00/48 h)										
2: Fresh extract (FE)	55.1± 1.6	(235)	17.4±1.9	(28)	0.001	9.4±0.5	(235)	2.7±0.5	(18)	0.001
3: FE+47° C, 30 min	61.8± 8.9	(5)	6.2±2.3	(6)	0.001	11.2±2.0	(5)	2.4±1.3	(6)	0.01
4: FE+100° C, 10 min	54.3± 6.1	(6)	48.7±9.3	(5)	–	6.1±1.4	(6)	5.5±1.8	(5)	–
5: FE+22° C, 24 h	60.4±16.0	(4)	14.9±6.5	(6)	0.02	7.7±1.5	(4)	2.8±1.4	(6)	–
6: FE+lyophilization, 1 week	46.9±11.4	(6)	11.8±3.2	(5)	0.05	3.7±1.3	(6)	2.2±1.4	(6)	–
7: FE+lyophilization, 4 week	44.8± 5.5	(18)	5.0±1.0	(6)	0.001	5.4±0.8	(18)	2.0±0.9	(6)	0.05
8: FE subcutaneous	54.0± 6.9	(6)	17.2±2.9	(7)	0.001	7.0±1.1	(6)	3.8±1.0	(6)	–
9: FE 1.0 mg/gbw	54.3± 6.1	(6)	6.1±1.4	(10)	0.001	6.1±1.4	(6)	0.9±0.5	(10)	0.001
FE 0.5 mg/gbw			9.7±2.0	(6)	0.001			2.3±0.8	(6)	0.05
FE 0.25 mg/gbw			32.1±5.7	(5)	0.05			3.6±1.3	(5)	–

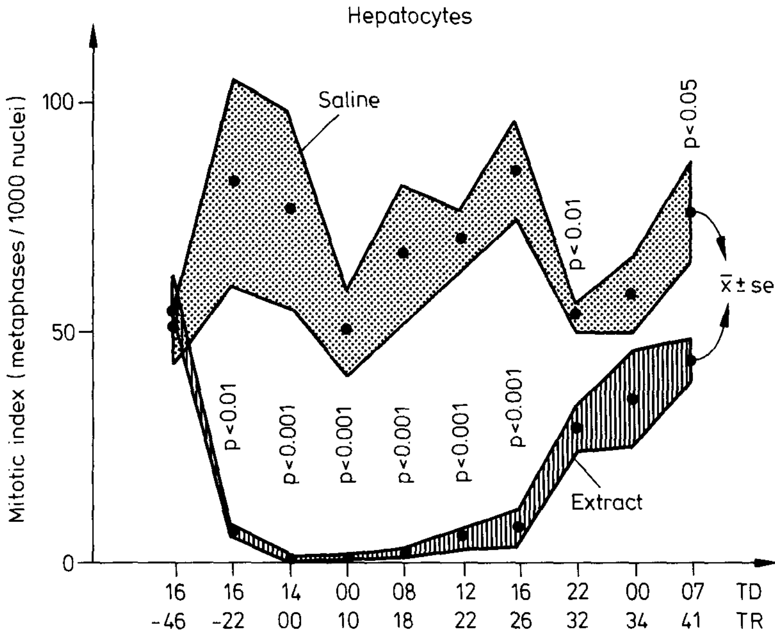

Fig. 2. Effect of tumor extracts on the mitotic activity of hepatocytes at the first peak during liver regeneration (12/46 = time of day (TD)/time of regeneration (TR)). The extract was injected in the same dose at different times from 46 h before partial hepatectomy to 41 h after hepatectomy. The dashed curve shows the results from the extract-injected animals and the dotted curve the results from the saline-injected animals. The dotted and dashed areas indicate the mitotic index as black, filled-in circles ±SEM. The statistical significance of the differences at each time point are also given

regenerating liver. The effect was not modified by exposing the extracts to temperatures of 47° C for 30 min or 22° C for 24 h although 10 min of boiling stopped the inhibiting effect. Lyophilization and storing at −18° C for 1–4 weeks did not modify the effect. The effect was also measurable when the extract was injected subcutaneously. The effect diminished when the dose was lowered, revealing an almost linear dose-response curve.

In general, the littoral cell population reacted like the hepatocytes, but here most of the differences were not significant.

Discussion

Our experiments have shown that aqueous extracts of an undifferentiated, fast-growing hepatoma have a significant inhibiting effect on the mitotic activity of regenerating hepatocytes, and only a weak inhibitory effect on the sinusoid littoral cell population. This inhibition can only be seen when partial hepatectomy is performed at a particular time of the day, and when the extract is injected during a particular time period in relation to the hepatectomy

An extract-induced inhibition of the regenerative process can be due to a single factor in the extract, or to a combination of different factors

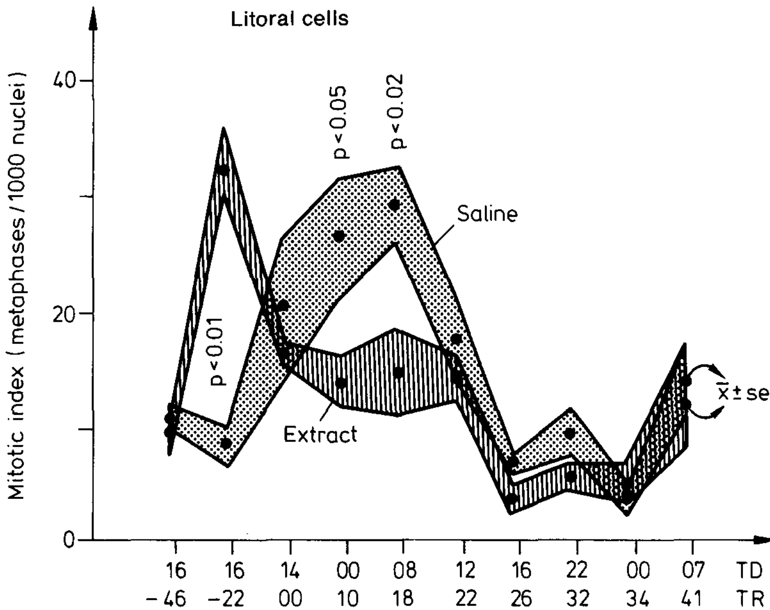

Fig. 3. Effect of tumor extracts on the mitotic activity of littoral cells at the first peak during liver regeneration (12/46 = time of day (TD)/time of regeneration (TR)). The extract was injected in the same dose at different times from 46 h before partial hepatectomy to 41 h after hepatectomy. The dotted curve shows the results from the saline-injected animals, and the dashed curve the results from the extract-injected animals. The dotted and dashed areas indicate the mitotic index as black, filled-in circles ±SEM. The statistical significance of the differences at each time point are also given

in the extract. One or more factors in the extract could also be working in combination with one or more factors in the body of the recipients. The effect can be produced by a specific factor or factors, or by non-specific factors, and it can be direct or indirect.

We have not been able to start biochemical procedures to try to purify the possible endogenous inhibitor obtained from this malignant tumor. Experiments to test for tissue specificity are also needed. A definition at the molecular level should be the goal.

However, the effect of the hepatoma extract is similar to reported effects of extracts obtained from normal liver. Growth-inhibiting effects of liver extracts were reported many years ago (Saetren 1956). Inhibiting and stimulating effects on liver regeneration, caused by tissue or tumor extracts, have since been reported by others (Iversen 1981). Some papers have reported stimulating effects of tissue extracts (Doljanski et al. 1944; Pashkiss 1958; Malmgren 1956), and some have reported inhibiting effects (Malmgren and Mills 1961; Terayama and Sasada 1968; Burns 1969). Since the methods of preparation and the methods of assay differ considerably in the various reports, it is difficult to compare the results.

In this paper we have demonstrated strong inhibition of the mitotic

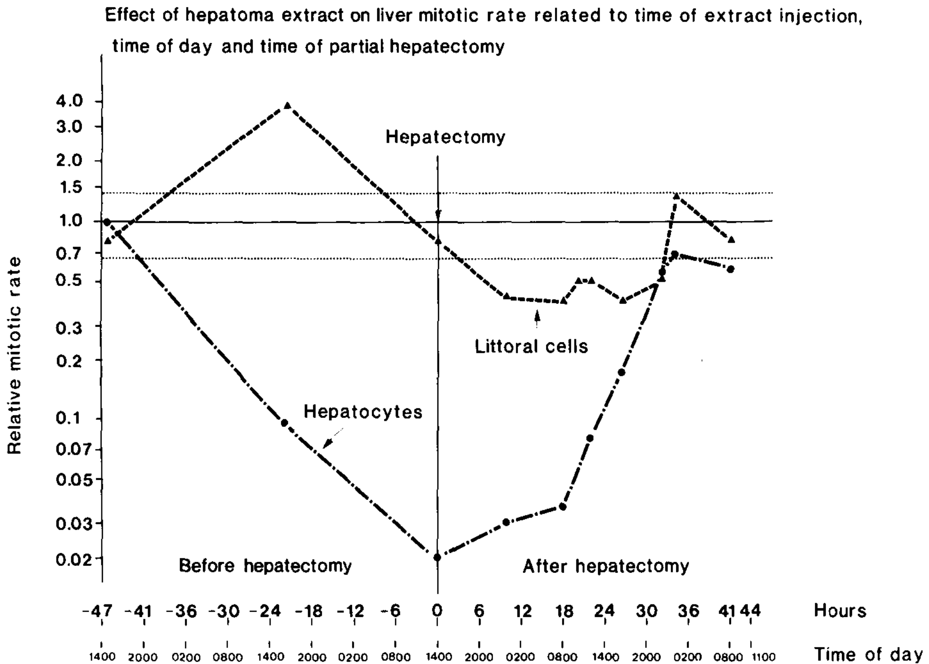

Fig. 4. Relative effect of hepatoma extract on liver mitotic rate related to time of extract injection, time of day, and time of partial hepatectomy. All values are related to results of saline injection, pooled together, and put at 1.00. The fine horizontal dotted lines above and below the abscissa indicate ± 2 SEM. The upper curve shows the results observed in the littoral cells at the first regenerative peak of mitotic rate, and the bottom curve shows the same results observed in the hepatocytes.

activity in regenerating liver caused by an extract of an undifferentiated, fast-growing hepatoma. The effect can be detected after a maximum lag of 60 h after the injection, and suppresses the first regeneratory peak of mitotic activity following partial hepatectomy. In a separate study we have also assayed about 15 more hepatomas (Echave Llanos et al., unpublished), and found most of the extracts to have inhibitory effects. No correlation has been found between the histologic type and the growth rate of the tumor on the one hand, and the inhibiting effect of their extracts on the other.

Similar effects of tumor extracts have been demonstrated quite recently from human rhabdomyosarcomas (Fryling et al. 1985; Iwata et al. 1985), and from a human colon carcinoma cell line (Levine et al. 1985).

The demonstrable effect of endogenous growth inhibitory substances (chalones) also seems to depend on the actual rate of proliferation in the tissue studied. Epidermal chalones, for instance, do not act in embryonic or post-natal skin (Bertsch and Marks 1978; Marks 1976; Elgjo and Cromarty 1977), or very effectively in rapidly regenerating skin (Bertsch et al. 1976), they also act at different strengths at different times of day (Echave Llanos et al. 1967a; Clausen and Elgjo 1984). This latter fact is probably

related to the well known and quite pronounced circadian rhythm in the rate of DNA synthesis and mitotic activity in many organs.

Based on the time pattern of the reactions, we believe that the effect of the extracts is one which takes place in the late G_1 and perhaps in the G_0 phases of the cell cycle. Since the effect diminishes just when DNA synthesis is increasing after partial hepatectomy, our findings indicate that the extracts contain one or more substances which inhibit cell flux at the G_1/S transition. This agrees with other reports, for example in mouse epidermis (Elgjo et al. 1981).

Both hepatocytes and littoral cells were affected. The extract, however, was produced from a tumor that contained malignant hepatocytes, littoral cells, stromal cells, inflammatory cells, vessel wall cells, etc., and it is possible that the extract contained various more or less tissue-specific substances.

We found a suitable period of time in relation to hepatectomy for the injection of the extract, but could not narrow it down further. The suitable period does not seem to depend on the circadian time, since injections of the extract during the resting (light) and the active (dark) periods are both equally good times for treatment.

We believe that the effect demonstrated is real and reproducible, and that it may be of biological significance. We hope that other laboratories will continue to search for endogenous growth-inhibitory principles in various organs and tumors. The success in finding stimulatory growth factors has the disadvantage that almost all the effects have been demonstrated in cell cultures only, and have not been confirmed in vivo. The growth-inhibitory effects of endogenous factors, on the other hand, are mostly demonstrable in vivo or in organ culture. Biological common sense suggests that growth regulation is probably a complex interplay between stimulatory and inhibitory factors, which balance each other. The inhibitory factors ought to be as important as the stimulatory ones.

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