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Interactive regeneration of liver and growth of Ehrlich ascites tumor in mice

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Adult mammalian liver cells multiply rapidly and enable regeneration of the organ following damage or partial hepatectomy (PH). Previously, we observed that implantation of Ehrlich ascites tumor (EAT) cells to mice immediately after 35% hepatectomy caused an increase in liver regeneration and labeling index. Growth of the EAT cells in mice is characterized by an initial exponential phase followed by a plateau phase 9–10 days after inoculation. It is also known that the ascitic fluid, formed during the growth of tumor, affects the multiplication of the cells so regenerative growth of the liver can have a selective effect on tumor growth. The main aim of the present study was to investigate how these two types of growth (regenerative and tumoral) could affect each other. In this study the liver regeneration percentage and multiplication of tumor cells on different days of mice (*Mus musculus*), to which 3×10^6 EAT cells were implanted immediately after the application of 57% PH, were investigated. According to the results obtained from the experiments, it is suggested that the stimulating effect on regeneration percentage of tumor seen originated either from only EAT tumor or from a synergistic impact of tumor together with stimulatory factors in regenerative growth. However, as a result of the presence of agents, such as PH ratio, tumor's variety, humoral factor and human hepatocyte growth factor from growth factors, singly or more than one together, regenerative growth stimulates the multiplication of EAT cells in the same host.

Key words: mouse, liver regeneration, Ehrlich ascites tumor, regeneration percentage, tumor growth.

Abbreviations: Ehrlich ascites tumor, EAT; human colon cancer, HCC; human hepatocyte growth factor, hHGF; partial hepatectomy, PH.

Introduction

Adult mouse liver hepatocytes are differentiated cells in Go phase under normal conditions and can execute functions important to the organism; the mitotic index of such cells is very low (1/1000).

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However, when partial hepatectomy (PH) is applied to the liver of adult mouse, the cells gain their ability to divide and start to multiply (HIGGINS & ANDERSON, 1931; WILSON et al., 1953; BUCHER, 1963; FAUSTO, 2001). This regenerative growth of the liver continues until the organ reaches its original volume. ALTUN (1996) determined that following PH of 35%, regeneration occurred rapidly until the third day and then the speed decreased during the following days. In another study (ALTUN & ÖZALPAN, 1998) where PH of 57% was applied, it was observed that regeneration was more rapid than in 35% PH, i.e. the rate of regeneration appeared to be proportional in the degree of PH. Interestingly, it has been reported that a humoral factor starts the liver regeneration and that this factor can also stimulate growth of a normal liver and even that of some tumors reached via blood circulation (PASCHKIS et al., 1955; MOOLTEN & BUCHER, 1967; SAKAI, 1970).

Ehrlich ascites tumor (EAT) cells stemmed from breast carcinoma and used frequently as model cancer cells are propagated by implanting into the peritoneal cavity of mice. In these cells, a multiplication phase, where the cell number increases exponentially, is observed immediately after implantation (TANNOCK, 1969; ALTUN, 1996). A plateau phase, where the number of cells stays almost stable, follows the exponential phase. In studies involving implantation of 3×10^6 cells into the peritoneal cavity, it was observed that the cell number increased exponentially up to the 9^{th} day and then the cells entered the plateau phase (TANNOCK, 1969; ALTUN, 1996). In parallel to the slowing down of multiplication, ascitic fluid would start to accumulate among EAT cells and the cells enter the plateau phase (TANNOCK, 1969; ALTUN, 1996).

By investigating the effect of four different tumors, such as reticuloendothelioma, fibrosarcoma and two breast cancers on normal liver, ANNAU et al. (1951) observed that an increase occurred in the weight of the livers and in the mitotic activity of mice and rats bearing tumors. A similar phenomenon was observed by MORGAN & CAMERON (1973). The latter study reported that in mice implanted with H6 hepatoma cells, liver weight, liver DNA content and synthesis increased (MORGAN & CAMERON, 1973). Other studies showed that alkaline phosphatase activity (KOJIMA & SAKURADA, 1976) and protein synthesis in liver (PAIN et al., 1984) increased in normal mice bearing EAT cells. MIYAZAKI et al. (1995) reported that in the rats with 70% PH and undergoing both ileocecal and transverse colon removals, liver regeneration and protein synthesis were inhibited between the second and seventh days, but from the tenth day, this inhibition was alleviated.

PASCHKIS et al. (1955) investigated the relationship for various tumors and found that hepatoma and Walker 256 tumors grew better in hepatectomized mice, but no change could be observed in tumors, such as Jensen sarcoma and Murphy lymphosarcoma. Since there was no apparent relation between unilateral nephrectomy or fracture of hind limbs, it was proposed that the humoral factor appearing during the regenerative growth acted selectively (PASCHKIS et al., 1955). In studies involving implantation of Walker 256 tumor (FISHER & FISHER, 1959), Yoshida sarcoma (ICHIHASHI et al., 1984), DHD-K12-TR strain colon adenocarcinoma (VAN DALE & GALAND, 1988), VX2 carcinoma (TANAKA, 1988), AH130 and Walker 256 cells (ASAGA et al., 1991), MC28 sarcoma cells (LOIZIDOU et al., 1991), 109A ascites hepatoma cells (UEDA et al., 1993), CC531 colon carcinoma (DE JONG et al., 1995), C26 colorectal hepatic metastases (DRIXLER et al., 2000) and Morris hepatoma (ZAGER et al., 2003) into animals with PH, it was found that either tumor growth was stimulated or metastasis numbers increased. In contrast, ONO et al. (1986) observed that in animals implanted with mouse ascites hepatoma MH134, plasmocytoma X-5563 and EAT cells, tumor growth was not affected by PH; however, by the time of examining the dependence of implantation of MH134 cells, tumor retardation could be seen in animals with PH. BARBEITO et al. (2001) investigated the effect of PH on the proliferation of hepatoma ES12a in mice, and observed that a decrease occurred in the tumor mitotic indices.

There are very few studies concerning the regenerative growth of the liver in the presence of tumoral and regenerative growths in the same host. ROSENE (1968) determined by the injection of EAT cells and reticulum cell sarcoma into the spleen of mice with PH that mitotic indices of tumor and liver cells increased. On the other hand, UEDA et al. (1993) observed that when AH109A ascites tumor was implanted subcutaneously and also into the liver of rats with PH, a significant increase occurred only in the tumor dimension of the liver. In addition, the same investigators observed increases in the amount of DNA synthesis and tissue blood flow in the liver (UEDA et al., 1993). ALTUN (1996) injected EAT cells into mice with 35% PH and found that while an inhibition formed in EAT cells only on the tenth day, an increase oc-

curred in percentage of the liver regeneration and LI. The effect of hepatocellular carcinoma ES12a on the hepatocyte mitotic peak after the PH (70%) was also investigated (BARBEITO et al., 2002).

The aim of the present study was to investigate the possible interaction between the liver regeneration and growth of EAT cells in mice with 57% PH.

Material and methods

Animals

Inbred male albino mice (*Mus musculus*) of strain BALB/C, 20–28 g in weight were used in this study. All experimental animals were 2.5-month-old and fed on their usual pellet (Hipodrom Ltd.) and water *ad lib*. Measurements of both liver regeneration percentage and growth of EAT cells were performed on five groups corresponding to days 1, 2, 3, 5 and 10 after PH.

Partial hepatectomy and liver regeneration percentage

For the study of regenerative growth, a PH of 57% was performed by removing the left lateral and median lobe of the liver of mice under ether anesthesia (HIGGINS & ANDERSON, 1931). The preoperative body weight and the amount of liver removed were determined for each animal.

The mice inoculated with tumor cells after PH (see next section) were sacrificed by cervical dislocation on the days specified above and their postoperative body weights were determined. The whole liver was then removed and weighed.

The average total liver weight (y) in a given group of mice was calculated by using the following linear equation modified by ALTUN (1996) from the work of HIGGINS & ANDERSON (1931):

$$y = 0.0783x - 0.624 \pm \frac{0.1293}{\sqrt{n}}$$

where x is the average body weight; and n the number of animals. The body weights determined experimentally were inserted into the equation, and the amount of total liver was calculated. Then, by subtracting the amount of liver removed during the operation from this value, the weight of the regenerated liver was found. From the amount of liver present at time of death for each group, the amount of the liver regeneration was subtracted and thus the hepatic increase was determined in grams. The regeneration percentage for each group was calculated according to GRISHAM (1960) using the formula below:

Regeneration (%) = 100 ×
× (weight of liver after regeneration −
− weight of liver remaining)/weight of liver remaining

The growth rate of EAT cells

Hyperdiploid EAT cells were produced by transplantation from mouse to mouse routinely every twelve days. For the transplantation of tumor cells, the fluid containing the tumor cells in mouse's peritoneal cavity was driven by a sterile injector and by diluting with Hank's suspension (HBSS; Difco Lab.), a suspension of 10^7 cell/mL was obtained. The cells were first tested in viability (PHILLIPS, 1973) and then $3x10^6$ cells were implanted to each animal with PH immediately after the surgery.

The implanted animals were sacrificed with servical dislocation on the days specified above. Peritoneal cavity of mice was opened and by washing with HBSS all tumor cells in the cavity were collected. Trypan blue was used to determine the viability and by counting the living cells on a hemocytometer the total number of EAT cells was determined for each mouse.

Statistical analysis

All quantitative data are presented in the text as means ± standard errors (SE). For the liver regeneration, multiple regression analysis was used to compare the correlation of the two groups. Statistical significance of the tumor cell numbers was determined with Student's t-test.

Results

Liver regeneration percentage

Without EAT cells. The time course of liver regeneration in mice with 57% PH is shown in Table 1. The livers of animals, which remained at 0.60 g, reached 1.06 g after one day of surgery (regeneration percentage = 76.2). At the end of second day following the surgery there was 0.45 g of hepatic increase (regeneration percentage = 81.1%). The remaining part of the liver of the third group, which was 0.61 g, reached 1.14 g at the end of the third day (regeneration percentage = 85.3%). On average, 0.64 g of liver remaining after the surgery, reached 1.26 g after 5 days of regeneration (regeneration percentage of liver was 96.8%). In the final group (10^{th} day), the average hepatic increase formed was found to be 0.69 g and regeneration percentage 104.9%.

With EAT cells. The time course of liver regeneration in mice implanted with 3×10^6 EAT cells immediately following 57% PH is shown in Table 2. On average, 0.49 g of liver remaining after the surgery, reached 0.85 g after one day of regeneration and tumoral growth. Then in one day, an increase 0.36 g of occurred corresponding to a regeneration percentage of 74.7%. The remaining part of the liver of the second group, which was 0.60 g, reached 1.11 g at the end of the second day (0.51 g hepatic increase, regeneration percentage

Table. 1. Data obtained by experiments and calculations from the mice after 57% partial hepatectomy (±SE).

		Before Regeneration					After Regeneration				
Number of animals	Time (day)	Body weight (g)	Liver[a] weight (g)	Ratio of liver to body weight (%)	Liver removed (g)	Liver remaining (g)	Body weight (g)	Liver weight (g)	Hepatic increase (g)	Ratio of liver to body weight (%)	Regeneration (%)
12	Cont.	24.30 ±0.35	1.45 ±0.05	5.93							
3	1	26.23 ±0.27	1.43 ±0.08	5.45	0.83 ±0.03	0.60 ±0.08	23.38 ±0.12	1.06 ±0.01	0.46 ±0.08	4.53	76.2
7	2	24.37 ±0.52	1.28 ±0.05	5.27	0.73 ±0.04	0.55 ±0.06	22.79 ±0.61	1.00 ±0.06	0.45 ±0.09	4.39	81.1
4	3	24.06 ±0.61	1.26 ±0.07	5.24	0.65 ±0.02	0.61 ±0.07	23.29 ±0.69	1.14 ±0.04	0.52 ±0.08	4.89	85.3
6	5	24.40 ±0.73	1.29 ±0.05	5.27	0.64 ±0.02	0.64 ±0.06	21.60 ±0.15	1.26 ±0.08	0.62 ±0.10	5.87	96.8
4	10	24.38 ±1.56	1.29 ±0.07	5.27	0.62 ±0.03	0.66 ±0.07	22.51 ±1.69	1.36 ±0.06	0.69 ±0.09	6.02	104.9

[a]Calculated from the linear equation.

Table 2. Data obtained by experiments and calculations from the mice after 57% partial hepatectomy and EAT cells (±SE).

		Before Regeneration					After Regeneration				
Number of animals	Time (day)	Body weight (g)	Liver[a] weight (g)	Ratio of liver to body weight (%)	Liver removed (g)	Liver remaining (g)	Body weight (g)	Liver weight (g)	Hepatic increase (g)	Ratio of liver to body weight (%)	Regeneration (%)
5	1	22.46 ±0.55	1.14 ±0.06	5.05	0.65 ±0.05	0.49 ±0.07	19.42 ±0.65	0.85 ±0.09	0.36 ±0.11	4.39	74.7
3	2	24.28 ±1.35	1.28 ±0.08	5.26	0.68 ±0.07	0.60 ±0.10	22.65 ±1.53	1.11 ±0.15	0.51 ±0.18	4.90	85.1
4	3	25.28 ±1.20	1.36 ±0.07	5.36	0.78 ±0.05	0.58 ±0.08	24.51 ±1.31	1.20 ±0.13	0.62 ±0.15	4.90	106.8
4	5	23.51 ±0.90	1.22 ±0.07	5.18	0.67 ±0.02	0.55 ±0.07	24.28 ±1.35	1.36 ±0.09	0.81 ±0.12	5.60	146.6
6	10	25.01 ±0.73	1.33 ±0.05	5.33	0.87 ±0.11	0.47 ±0.12	26.49 ±1.16	1.34 ±0.09	0.87 ±0.15	5.05	185.3

[a] Calculated from the linear equation.

= 85.1%). At the end of the third day following the surgery, there was 0.62 g of hepatic increase, liver weight becoming 1.20 g (regeneration percentage = 106.8%). The livers of animals, which remained at 0.55 g, reached 1.36 g after 5 days (regeneration percentage = 146.6%). In the final group (10^{th} day), the average hepatic increase formed was found to be 0.87 g and regeneration percentage 185.3%.

As a result of implanting 3×10^6 EAT cells to mice immediately after 57% PH, rather rapid regenerative growth was accomplished in the liver. This regenerative growth was linear up to the fifth day and then the rate of regeneration slowed down. When these results are compared with those of our previous study (ALTUN & ÖZALPAN, 1998) where only 57% PH was applied (Table 1), it is observed that in the animals of both groups the regeneration percentages increased with time after surgery. The data revealed, that with the presence of tumoral growth besides the regenerative growth of the liver produced, no effect was seen in the first and the second days. Then, the regeneration increased rapidly and although the speed decreased a little after the fifth day, when the normal regenerative growth was completed, the growth continued. This difference observed in regeneration percentages has been shown to be significant ($P < 0.005$) by regression analysis.

Growth rate of EAT cells

In mice without PH. As a result of implanting 3×10^6 EAT cells to mice, the difference in multiplication of tumor cells depending on days are given in Figure 1. The number of EAT cells in mice implanted with 3×10^6 EAT cells increase rapidly and this number reaches 102 751 802 on the 5^{th} day and 816 513 673 on the 10^{th} day.

In mice with PH. The results of the experiment involving implanting 3×10^6 EAT cells to mice immediately after 57% PH and the difference observed in the multiplication of the tumor cells on days after surgery are also given in Figure 1. Exponentially multiplying EAT cells, in the presence of regenerative growth in the same animal, also grow exponentially. As can be seen in Figure 1, the number of EAT cells in 57% PH applied mice increase rapidly and this number reaches 83 967 500 on the 5^{th} day and 1 169 244 444 on the 10^{th} day.

As a result of implanting 3×10^6 EAT cells immediately after 57% PH, EAT cell number increased exponentially over 9–10 days after surgery. When these results are compared with the values from animals injected with only EAT cells (Fig. 1; circles), it was observed that both groups exhibited an exponential growth. It can also be seen (Figure 1) that animals with EAT (circles) and those with both EAT and PH (triangles) exhibit differences in cell numbers after the 3^{rd} day. This increase observed in cell numbers from the 3^{rd} day on is higher in the animals implanted with EAT following 57% PH application than those containing only EAT cells. This difference observed in the viewpoint of cell counts was found to be significant at $P < 0.005$ level on the 3^{rd} and 5^{th} days, whereas on the 10^{th} day at $P < 0.05$ level. In this way, the increase in EAT cells at such a significant level was determined to be due to regenerative growth as a result of 57% PH application.

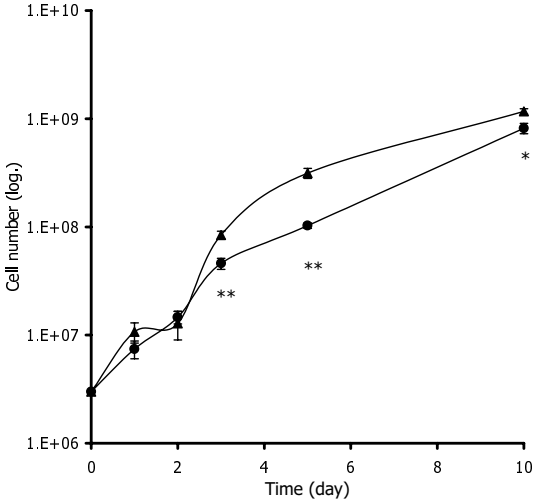

Fig. 1 The growth rate in mice of EAT (●) and PH+EAT (▲). Data are presented as mean ± SE. (*) indicates $P < 0.05$ and (**) indicates $P < 0.005$.

Discussion

In the literature, ROSENE (1968) used implanted EAT and reticulum sarcoma tumor cells into the spleens of mice with almost 30% PH. The results of this investigation showed increased mitotic indices of both tumor and liver cells. ONO et al. (1986) found that in the case of implanting mouse with ascites hepatoma (MH134), plasmocytoma (x-5563) and EAT cells after PH, x-5563 and EAT were not affected by PH. Furthermore, when MH134 cells were given 3–7 days after PH, retardation and rejection were observed following tumor growth; as a result of implantation prior to 7–15 days from PH, a retardation in the tumors was not observed (ONO et al., 1986). ALTUN (1996)

applied 35% PH to mice and when 3×10^6 EAT cells were implanted into the same animals immediately post surgery, just as in the present study, even though the multiplication of tumor cells was inhibited, a stimulating effect was observed in the liver regeneration and labeling index. UEDA et al. (1993) investigated the relation between AH109A ascites hepatoma cells implanted both subcutaneously and into liver of rats 5 days prior to PH, and regenerative growth. They observed increases in tissue blood flow of animals with PH in parallel to the increase in the amount of DNA synthesis of hepatocytes, liver's internal tumor and subcutaneous tumor cells. It was suggested that this result occurred due to the increase of some humoral hepatotrophic factors in both livers and tumor cells (UEDA et al., 1993). BARBEITO et al. (2002) determined that the peak of liver's mitotic activity in the mice, carrying hepatocellular carcinoma ES12a and with 70% PH, took place fourtimes earlier than in non-tumor-bearing mice.

Some studies showed that tumors are effective on the livers of unoperated normal animals. In the livers of rats and mice having reticuloendothelioma, fibrosarcoma and two mammary tumors, growth and mitotic activity increases were also found (ANNAU et al., 1951). However, MORGAN & CAMERON (1973) found that in mice with H6 hepatoma, liver's DNA synthesis and amount increased and this increase originated from the tumor. KOJIMA & SAKURADA (1976) found that liver alkaline phosphatase activity in EAT-bearing mice increased due to the effect of some factor(s) secreted by the tumor. PAIN et al. (1984) recorded that, again in EAT-bearing mice, amounts of liver protein synthesis and, in consequence, body weight increased, but there was no increase in the gastrocinemius muscle of the same animals. The investigators stated that tumoral growth affected host animal's protein metabolism.

After applying 67% PH to different rat strains, PASCHKIS et al. (1955) transplanted different tumors to the animals and investigated the relations between regenerative growth and tumor growth. In their study, they observed that hepatoma and Walker 256 tumors grew better, but no change occurred in Jensen sarcoma and Murphy lymphosarcoma. The investigators could not find a relation between these growths and tumors when the same experiments were repeated with growing types of both unilateral nephrectomy and fracture of the hind limbs. They suggested that a humoral factor appearing during the regenerative growth caused this process and that this factor behaved selectively (PASCHKIS et al., 1955). ICHIHASHI et al. (1984) reported that when Yoshida sarcoma was transplanted into the remaining part of the liver of rats with 70% PH, tumor growth was stimulated in a significant way and this originated from liver regeneration. The relation between AH130 and Walker 256 tumors and regeneration was investigated both *in vivo* and *in vitro* (ASAGA et al., 1991). By transplanting the tumor cells to rats subcutaneously on the same day with PH, it was found that tumor growth in PH-applied animals was larger in comparison with controls, and in the case of tumor transplantation 7 days post PH application, the increase in growth was lower. In the present study, it is also pointed out that the humoral factor, which appeared during the regenerative growth, produced this effect. It has been determined that MC 28 sarcoma cells developed with methylcholanthrene at 2/3 ratio when given intraportally to mice applied with PH ($\sim$ 67%), sarcoma cells grew better (LOIZIDOU et al., 1991). DE JONG et al. (1995) reported that in the liver's remaining part of rats, to which 70% PH was applied, CC 531 colon carcinoma was stimulated and any effect in the weights of extrahepatic tumor did not occur. The effect of PH on the proliferation of hepatoma ES12a in mice was also investigated (BARBEITO et al., 2001). It was reported that tumor growth in mice with PH decreased from first day to third day after surgery, and this effect in the third postoperative day was induced by liver regeneration. ZAGER et al. (2003) investigated the effect of hepatic regeneration on the growth of Morris hepatoma in the liver and the rate of DNA synthesis in both the liver and tumor. They showed that liver regeneration enhanced liver and tumor DNA synthesis and that hepatectomy increased the growth of Morris hepatoma in rat's liver (ZAGER et al., 2003).

It has been determined that when human colon cancer (HCC) was injected subcutaneously to nude mice with PH, the time of their measurable dimension was smaller than controls and their growth speed was higher (GUTMAN et al., 1994-1995). When the same mitotic index was overlooked, it was observed that it reached the maximum value on day 2, just as in the regenerated liver. On the other hand, GUTMAN et al. (1994–1995) indicated that this rapid growth in the tumor was not observed after liver regeneration's completion. The investigators reported that this rapid growth, which was not observed in human melanoma, colon, prostate and renal cancers, was only unique to HCC and the stimulation developed on account of growth factor present in the blood circulation during liver regeneration.

Possible nature of liver-specific growth factor

In the peritoneal fluids of humans (cirrhosis and non-cirrhosis patients with a majority hepatocellular carcinoma) to which hepatectomy was applied, a factor named "human hepatocyte growth factor" (hHGF) which increased DNA synthesis of rat hepatocyte cultures was found (Miyata et al., 1996b). Miyata et al. (1996a) reported that the amount of hHGF formed in the sera of cirrhosis and non-cirrhosis patients, as a result of PH surgery, increased in direct ratio to the liver's weight, and even patients' damaged tissue's level could be determined depending on the amount of hHGF. Fausto (2001) reported that growth factors, such as hepatocyte growth factor, transforming growth factor-α and epidermal growth factor, are found in the liver, and both hepatocyte growth factor and transforming growth factor-α levels increase after PH. Besides these, along with several inhibiting factors, effective in liver regeneration after PH application, it was reported that many stimulating factors are also secreted locally or from other parts of the body (Wu et al., 1998).

When the available data on the relation between different tumors and different hepatectomy ratios for various genera and species of animals are taken together, it can be concluded that tumor implantation has a stimulating impact on both normal and regenerating liver. In studies where different tumors and animals were used, as Paschkis et al. (1955) pointed out, regenerative growth's selective effect on tumors appeared. The results of our study qualitatively support this suggestion.

In conclusion, it is assumed that the stimulatory effect on regeneration percent of tumor originated either from only EAT tumor or from a synergistic impact of tumor together with the stimulating factors in regenerative growth. However, as a result of the presence of factors, such as PH ratio, tumor's variety, humoral factors and hHGF, single or more than one together, regenerative growth stimulates the multiplication of EAT cells in the same host.

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