# EFFECT OF CHALONE ON HEPATOCYTE PROLIFERATION IN REGENERATING LIVER OF MICE OF DIFFERENT AGES

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Regeneration of the liver after partial hepatectomy (PH) varies in its kinetic characteristics, depending on many factors [3, 9-11]. This explains the importance of analysis of the controlling action of chalone in relation to the dynamics of the proliferative response. For this purpose the effect of repeated injection of chalone on hepatocyte proliferation was studied in the regenerating liver of two groups of mice differing in age and in the conditions of PH.

## EXPERIMENTAL METHOD

Male (CBA × C57BL/6)$F_1$ mice aged 1-1.5 months and weighing 14-16 g, and aged 3 months and weighing 20-22 g, were used. PH was performed by the method of Higgins and Anderson on young mice between 10 and 11 p.m. and on mature mice between 9 and 10 a.m. The ethanol fraction of aqueous extract of rabbit liver, conventionally called chalone in this paper, was obtained by Verly's method [14]. It was injected intraperitoneally, in a dose of 10 mg in 0.1 ml of physiological saline each time. The scheme of administration is given in Tables 1 and 2. Control mice received 0.1 ml of physiological saline. Repeated injections of chalone were used to maintain its concentration constant, for it has been shown that the action of this substance after a single injection is of short duration [2]. The mice were killed at various times after PH — from 30 to 48 h. The mice were given an injection of [$^3$H]thymidine in a dose of 1 μCi/g body weight (specific radioactivity 14 Ci/mmole) 30 min before sacrifice. The index of labeled nuclei (ILN) and mitotic index (MI) were determined by counting 3000-5000 hepatocyte nuclei in sections with autoradiographs. Nuclei in the $G_2$-phase ($I_{G_2}$) and postmitotic nuclei (post-MI) were counted on the basis of structural differences between mouse hepatocyte nuclei [8, 12], by which premitotic [13] and postmitotic [4, 6] states could be distinguished. Two categories were distinguished among the postmitoses: early, with a post-telophase structure (post-MI I), and later (post-MI II). To make sure that $G_2$-nuclei isolated on the basis of morphology and postmitoses are in fact nuclei which have passed through the phase of DNA synthesis, an experiment was carried out in which the total number of hepatocyte nuclei in the mouse liver stimulated by the operation to synthesize DNA was established by the direct method — by counting labeled nuclei after repeated injection of [$^3$H]thymidine (every 4 h, starting from 20 h after PH). Nuclei in the S phase at the time of sacrifice (24-48 h after PH) were eliminated by injecting [$^{14}$C]thymidine in a dose of 5 μCi per mouse. It will be clear from Table 3, which gives the results of this particular experiment, that the index of [$^3$H]thymidine-labeled hepatocytes agreed well with the total of the proliferation indices based on morphology, i.e., $I_{G_2}$, MI, ILN, and post-MI. Within a limited time interval (1-2 days after PH) these indices do thus in fact characterize the population which has passed through the S phase before sacrifice of the animal.

## EXPERIMENTAL RESULTS

The most objective parameter of the dynamics of cell proliferation in the control series is the sum of the indices of proliferation (SI). As Table 1 shows, it fluctuates only slightly within the sample and increases regularly with the regeneration time. Other indices of

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TABLE 1. Effect of Chalone on Hepatocyte Proliferation in Regenerating Liver of Young Mice

| Experimental conditions | No. of mouse | ILN, ‰ | $I_{G_2}$, ‰ | MI, ‰ | Post-MI I, ‰ | Post-MI II, ‰ | SI, ‰ |
|---|---|---|---|---|---|---|---|
| | | | Group 1 (30 h of regeneration) | | | | |
| Control | 1 | 70 | 19 | 8 | 21 | 0 | 118 |
| | 2 | 149 | 63 | 7 | 16 | 0 | 235 |
| | 3 | 64 | 82 | 27 | 70 | 26 | 269 |
| | 4 | 190 | 33 | 40 | 40 | 0 | 303 |
| | | | Group 2 (34 h of regeneration) | | | | |
| | 5 | 202 | 27 | 13 | 89 | 158 | 489 |
| | 6 | 192 | 19 | 24 | 155 | 34 | 424 |
| | 7 | 244 | 29 | 2 | 111 | 67 | 453 |
| | | | Group 3 (40 h of regeneration) | | | | |
| | 8 | 299 | 114 | 4 | 47 | 189 | 653 |
| | 9 | 209 | 52 | 8 | 11 | 220 | 500 |
| | | | Group 4 (43 h of regeneration) | | | | |
| | 10 | 166 | 178 | 3 | 18 | 337 | 702 |
| | 11 | 86 | 143 | 3 | 28 | 354 | 614 |
| | 12 | 333 | 40 | 27 | 26 | 265 | 691 |
| | 13 | 339 | 125 | 10 | 10 | 208 | 692 |
| | 14 | 269 | 114 | 17 | 21 | 110 | 531 |
| | 15 | 314 | 100 | 8 | 30 | 198 | 650 |
| | 16 | 195 | 132 | 50 | 106 | 148 | 631 |
| | 17 | 206 | 86 | 8 | 45 | 229 | 574 |
| | | | Group 5 (30 h of regeneration)* | | | | |
| Chalone | 18 | 10 | 74 | 28 | 162 | 0 | 274 |
| | 19 | 11 | 60 | 10 | 49 | 47 | 177 |
| | 20 | 4 | 9 | 4 | 17 | 0 | 34 |
| | 21 | 2 | 3 | 1 | 2 | 0 | 8 |
| | | | Group 6 (43 h of regeneration) | | | | |
| | 22 | 254 | 23 | 1 | 3 | 90 | 371 |
| | 23 | 62 | 15 | 2 | 11 | 2 | 92 |
| | 24 | 24 | 8 | 0 | 0 | 51 | 83 |
| | 25 | 228 | 5 | 1 | 6 | 48 | 288 |

*Injection of chalone every 2 h starting with 16 h after PH.

TABLE 2. Effect of Chalone in Hepatocyte Proliferation in Regenerating Liver of Sexually Mature Mice

| Experimental condition | No. of mouse | ILN, ‰ | $I_{G_2}$, ‰ | MI, ‰ | Post-MI I, ‰ | Post-MI II, ‰ | SI, ‰ |
|---|---|---|---|---|---|---|---|
| | | | Group 7 (48 h of regeneration) | | | | |
| Control | 1 | 254 | 163 | 34 | 18 | 94 | 563 |
| | 2 | 169 | 164 | 14 | 17 | 29 | 393 |
| | 3 | 343 | 37 | 1 | 1 | 4 | 386 |
| | 4 | 314 | 35 | 3 | 1 | 5 | 358 |
| | | | Group 8 (48 h of regeneration)* | | | | |
| Chalone | 5 | 269 | 102 | 17 | 17 | 24 | 429 |
| | 6 | 157 | 75 | 9 | 8 | 26 | 275 |
| | 7 | 146 | 18 | 2 | 1 | 10 | 177 |
| | 8 | 137 | 59 | 11 | 5 | 53 | 265 |
| | 9 | 105 | 67 | 15 | 18 | 92 | 297 |

*Injection of chalone every 2 h starting with 20 h after PH.

proliferation available for consideration, taken separately are less definitely linked with this phase of the process. For instance, equal values of ILN were recorded after both 30 h and 43 h of regeneration. The relationship between the four parameters varies considerably within the sample. For example, in group 1, in mouse No. 2, the ratio of the number of labeled nuclei to the number of nuclei in the $G_2$ phase was 1:0.4, whereas in mouse No. 3 it was 1:1.3. This difference is evidently connected with the fact that regeneration proliferation

TABLE 3. Comparison of Autoradiographic and Morphologic Methods of Determining Total Number of Hepatocytes Which Have Passed through S Phase during 24-48 h of Regeneration (injection of [$^3$H]thymidine every 4 h starting with 20 h after PH)

| Mouse no. | ILN, ‰ ($^3$H-thymidine) | $I_{G_2}$ + ILN + post-MI, ‰ |
|---|---|---|
| 1 | 11 | 11 (100) |
| 2 | 88 | 88 (100) |
| 3 | 89 | 89 (100) |
| 4 | 332 | 343 (94) |
| 5 | 276 | 285 (94) |

Legend. % of labeled nuclei shown in parentheses.

started earlier in mouse No. 3 than in mouse No. 2. This is also shown by the high value of MI and the highest value of post-MI I in the group, and also by the fact that only in one mouse were post-MI II present 30 h after PH.

It will be clear from Table 1 that 30 h after the operation, among mice receiving repeated injections of chalone (group 5) considerable inhibition of proliferation was observed, and SI amounted to only 5-10% of the control value (mouse Nos. 20 and 21). In two other mice (Nos. 18 and 19) SI was not lower than in the control, but was composed mainly of $I_{G_2}$, MI, and post-MI. In all four mice of this group ILN was considerably lower than in the control. Toward 43 h of regeneration, during administration of chalone (group 6) a considerable (almost threefold) decrease in SI was observed on account of low values of $I_{G_2}$, MI, post-MI I, and post-MI II. If both times (30 and 43 h of regeneration) are examined it will be concluded that the effect of chalone in young mice on hepatocyte proliferation is manifested by a varied degree of inhibition of DNA synthesis; in some individuals this inhibition does not begin at the beginning — the first wave of S cells occurs successfully (mitoses, postmitoses, and nuclei in the $G_2$ phase at the time of sacrifice).

Table 2 gives the results of experiments on more adult, sexually mature mice. As Table 2 shows, in animals of the control group (seven) SI was formed mainly from ILN and $I_{G_2}$; the exception was mouse No. 1. The fact that a considerably higher proportion of cells was in the S phase (or in the S and $G_2$ phases) than the number of mitoses and postmitoses indicates that marked intensification of DNA synthesis took place in these animals shortly before sacrifice, i.e., considerably later than in young mice, in which considerable proliferation was being recorded as early as 30 h after PH. Judging from the values for group 8, the action of chalone on hepatocyte proliferation in adult mice was manifested as a decrease in ILN and $I_{G_2}$ by 33 and 36%, respectively; MI and post-MI did not differ from their values in the control. This means that the hepatocytes which were stimulated first to synthesize DNA were resistant in adult mice to the inhibitory action of chalone, which was exhibited later.

The kinetics of regeneration proliferation of mouse hepatocytes, studied by four interconnected parameters, enables the concrete details of the dynamics of the process to be worked out; by averaging of the data it appears to be rigidly determined, and to be characterized by either two or three waves [5, 7, 15]. The increase in SI with an increase in regeneration time, combined with considerable individual variability of the relative values of S, $I_{G_2}$, MI, and post-MI, are evidence that the proliferative process arising as a result of PH is discrete; it is formed by different numbers of hepatocyte populations, synchronized with respect to the S period, the so-called S pulses [4]. These synchronized populations, as will be seen, are sufficiently equal in size in young mice, but in adult mice they are initially much smaller than later. This difference, which is evidently connected with age and also with the time of day at which the operation was performed [1, 3, 10, 11], was detected only through the use of additional, nontraditional parameters such as $I_{G_2}$ and, in particular, post-MI of the hepatocytes. Without consideration of them, the dynamics of the process would be apparently identical in the groups of mice compared.

It can be concluded from these results that the variability of regeneration as a process of secondary development in the mouse liver is grouped in character: Samples differing in age

and conditions of operation respond quite uniformly to PH. By contrast, the response of animals to injection of chalone is not so definite. For instance, in some young mice, strong inhibition of proliferation takes place from the very beginning. In other mice of this group regeneration proliferation of hepatocytes was insensitive to chalone in the initial period, just as in all the mature mice. In the latter, the inhibitory effect of chalone was weaker in general.

Summing up the results, a common feature in the response of mice of both groups to chalone will be noted. Despite repeated injection of the chalone (throughout the period of regeneration studied) there was no complete suppression of DNA synthesis. The discrete character of proliferation remained undisturbed, with the "S pulse" of varied magnitude serving as the structural unit of the process. It is important to note that its magnitude did not exceed the control level (as shown by the value of ILN), i.e., the synchronization effect was absent (this must be tested at later times also). The facts described above serve as basis for the hypothesis that the controlling action of chalone on regeneration of the liver is manifested by a slowing of its course. Slowing of this kind may be regarded in this case as a more optimal variant of the regeneration process.

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