Polyamines are required for the initiation of rat liver regeneration

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A large number of studies applying inhibitors of polyamine biosynthesis have indicated that these compounds are required for animal cell proliferation. Here we show, using a transgenic rat model with activated polyamine catabolism, that a certain critical concentration of the higher polyamines spermidine and spermine is required for liver regeneration. Partial hepatectomy of transgenic rats expressing spermidine/spermine *N*¹-acetyltransferase (SSAT) under the control of mouse metallothionein promoter strikingly induced the enzyme at 24 h and reduced hepatic spermidine by 80 %. At that time, the weight of the liver remnant was significantly increased in syngenic rats and proliferating cell nuclear antigen (PCNA) labelling index was 20 %, whereas the transgenic rats showed no liver weight gain and their PCNA-positive cells accounted for 0.5 % of hepatocytes. Similarly, hepatic thymidine incorporation was markedly enhanced at this time point in syngenic, but not in transgenic, animals, whereas the rate of leucine incorporation was only marginally affected in

the transgenic animals. At 3 days after operation, the spermidine pool in transgenic livers had increased to the pre-operative level, the remnant weight was significantly elevated and hepatic PCNA labelling index increased to 5 %. *N*¹,*N*¹¹-Diethylnorspermine, a powerful inducer of SSAT, inhibited liver weight gain and proliferative activity in both syngenic and transgenic rats. We found an extremely close correlation between hepatic spermidine, and less close between spermine, concentrations and PCNA labelling index during early liver regeneration. These results indicate that spermidine and/or spermine, but apparently not putrescine, are required for liver regeneration, yet at concentrations smaller than those normally found after partial hepatectomy.

Key words: ornithine decarboxylase, proliferating cell nuclear antigen, spermidine/spermine *N*¹-acetyltransferase gene, transgenic rat.

INTRODUCTION

The polyamines putrescine, spermidine and spermine are small aliphatic cations the metabolism of which is closely connected to cell growth. Pharmacological intervention of their biosynthetic machinery almost always results in growth inhibition in animal cells [1]. However, the cellular function(s) of the polyamines are largely unknown in general and those of the individual polyamines in particular.

In attempts to elucidate the functions of polyamines, we have generated a number of transgenic mouse and rat lines with genetically altered polyamine metabolism. Overexpression of ornithine decarboxylase (ODC), the rate-controlling enzyme of polyamine biosynthesis, resulted in impaired spermatogenesis [2,3], partial resistance to physically and chemically induced seizures [4] in transgenic mice, and partial protection against cerebral ischaemia in transgenic rats [5]. Transgenic activation of polyamine catabolism through the overexpression of spermidine/spermine *N*¹-acetyltransferase (SSAT), the rate-controlling enzyme in polyamine degradation, brought about a plethora of phenotypic changes in transgenic mice, including hairlessness, female infertility, lack of subcutaneous fat deposits [6] and protection against kainate-induced toxicity [7]. Overexpression of SSAT under the control of the heavy-metal-inducible metallothionein I promoter in transgenic rats led to acute pancreatitis upon exposure of the animals to non-toxic doses of zinc [8].

In the present study we have subjected transgenic rats overexpressing SSAT under the control of mouse metallothionein I promoter to partial hepatectomy. The operation strikingly induced SSAT at 24 h post-operatively and profoundly depleted the hepatic spermidine pool. Under these conditions, the transgenic livers failed to initiate the regenerative process, which only

started later when spermidine concentration was increased to the pre-operative level.

MATERIALS AND METHODS

Generation of transgenic rats

The production of transgenic Wistar rats has been described in detail previously [8]. Partial hepatectomy (67 %) was carried out under midazolam anaesthesia by the original method of Higgins and Anderson [9].

Immunohistochemistry of proliferating cell nuclear antigen (PCNA)

PCNA was detected from formalin-fixed paraffin-embedded tissue sections. Sections of 4 µm were cut mounted on SuperFrost Plus glass slides (Menzel-Glaser, Freiburg, Germany), deparaffinized and rehydrated in a decreasing alcohol series. Non-specific protein binding was blocked by preincubation with 3 % BSA in PBS for 1 h. Slides were then incubated overnight with 1:200 dilution of FITC-conjugated mouse monoclonal anti-(human PCNA) antibody (Santa Cruz Biotechnology) at +4 °C. FITC-conjugated antibodies were detected with alkaline-phosphatase-labelled sheep anti-FITC antibodies (Boehringer Mannheim) diluted 1:1000 in 1 % BSA in PBS for 30 min at +37 °C. All antibody incubations were followed by three 5 min washes in PBS. Before substrate incubation the slides were washed for 5 min in alkaline phosphatase substrate buffer supplemented with 1 mM Levamisole (Sigma). The sections were then stained with Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate (Boehringer Mannheim) according to manufacturer's instructions for 30 min at ambient temperature. The reaction was stopped by washing the slides with 20 mM EDTA/1 mM levamisole in

Abbreviations used: SSAT, spermidine/spermine *N*¹-acetyltransferase; ODC, ornithine decarboxylase; DENSPM, *N*¹,*N*¹¹-diethylnorspermine; PCNA, proliferating cell nuclear antigen.

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PBS. The sections were counterstained slightly with Mayer's haematoxylin prior to mounting with Gurr® Aquamount (BDH). Negative controls were used to assess and control the staining procedure. To obtain the PCNA labelling index, a total of 550 cells were counted.

Analytical methods

Total RNA was isolated by the guanidinium isothiocyanate method [10]. Polyamines and their acetylated derivatives were determined with the aid of HPLC as described by Hyvönen et al. [11]. ODC and SSAT activities were assayed by published methods [12,13]. Thymidine incorporation was measured by injecting 10 μ Ci of [*Me*-³H]thymidine (specific radioactivity 82 mCi/mmol; Amersham Pharmacia Biotech) into the animals 30 min before they were killed and measuring radioactivity in trichloroacetic acid-insoluble fractions of liver homogenates. Leucine incorporation was measured by injecting 1 μ Ci of L-[U-¹⁴C]leucine (specific radioactivity 295 mCi/mmol, Amersham Pharmacia Biotech) into animals 2 h before they were killed and radioactivity was measured as described above.

Analysis of variance was used for statistical analyses. For multiple comparisons the method of Bonferroni was used (a software program Statview 4.0; Abacus Concept, Inc., Berkeley, CA, U.S.A.). For paired comparisons a two-tailed *t* test was used.

Chemicals

*N*¹,*N*¹¹-Diethylnorspermine (DENS PM) was synthesized as described previously [8]. The drug was dissolved in physiological saline and injected daily (50 mg/kg) starting 2 h after partial hepatectomy.

RESULTS

Partial hepatectomy induces hepatic polyamine catabolism in transgenic rats

As metallothionein I is known to be strikingly induced during early liver regeneration [14], the aforementioned transgenic rats were subjected to partial hepatectomy in order to elucidate the effect of enhanced polyamine catabolism on liver regeneration. Rat liver regeneration is one of the fastest growth processes in animal tissues; after the resection of two-thirds of the liver

Figure 1 Liver weight gain and proliferative activity in syngenic and transgenic rats without or with DENS PM treatment

The animals received saline or DENS PM (50 mg/kg) daily starting 2 h after partial hepatectomy. There were three or four animals in each group. The error bars represent S.D. ****P* < 0.001; statistical significance of the differences as compared with the 0 h groups. The initial liver weight for syngenic animals was 2.06 ± 0.26 g (100%) and that for the transgenic animals 2.46 ± 0.66 g (100%). (A) The weight gain of the liver remnant (%); (B) PCNA labelling index.

the original organ mass is restored in just 1 week, implying a growth rate of about 1 g/day. As shown in Table 1, normal rat liver regeneration is characterized by an early increase in ODC activity and a marked expansion of putrescine and spermidine pools, with a decrease in spermine content. The transgenic

Table 1 Hepatic ODC and SSAT activities and polyamine pools in partially hepatectomized syngenic and transgenic rats without or with DENS PM treatment

There were three or four animals in each group. Values are means $\pm$ S.D. Abbreviation: N.D., not detected. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, significance of the differences between transgenic and syngenic animals.

Group/time after partial hepatectomy (h)	ODC (pmol/30 min per mg of tissue)	SSAT (pmol/10 min per mg of tissue)	[polyamine] (pmol/mg of tissue)			
			Putrescine	N ¹ -Acetylspermidine	Spermidine	Spermine
Syngenic						
0	N.D.	N.D.	N.D.	N.D.	992 ± 240	846 ± 24
24	58.7 ± 24.7	N.D.	166 ± 36	44 ± 16	1815 ± 97	554 ± 7
24 + DENS PM	257 ± 119	2.3 ± 4.0	1460 ± 512	11 ± 14	940 ± 255	402 ± 23
72	14.3 ± 3.2	0.41 ± 0.71	65 ± 6	N.D.	2250 ± 196	603 ± 79
72 + DENS PM	8.2 ± 3.9	N.D.	51 ± 11	N.D.	2130 ± 46	470 ± 23
Transgenic						
0	69.1 ± 5.7***	16.9 ± 10.2**	1540 ± 307**	29 ± 15	1110 ± 196	75 ± 13**
24	121 ± 84	3910 ± 3350	1570 ± 161***	29 ± 5	243 ± 30***	43 ± 7***
24 + DENS PM	347 ± 190	122500 ± 360**	3020 ± 1590	75 ± 20	149 ± 14**	30 ± 3**
72	224 ± 53**	776 ± 1462	2210 ± 184***	19 ± 7*	924 ± 105***	68 ± 14***
72 + DENS PM	132 ± 36**	3040 ± 2587	1750 ± 190***	30 ± 5**	312 ± 33***	4.6 ± 2***

Figure 2 Immunohistochemistry of PCNA at 24 h after partial hepatectomy in liver sections from syngenic (A) and transgenic (B) animals

The immunohistochemistry of PCNA was carried out as described in the Materials and methods section and the liver sections were counter-stained with haematoxylin. The animals were from the thymidine-incorporation experiment described in the text. Magnification, $\times 160$.

animals already showed all the signs of activated polyamine catabolism before partial hepatectomy. Putrescine was the major polyamine and, interestingly, normal spermidine concentration appeared to be maintained at the cost of spermine, the pool of which was decreased to less than one-tenth of that in normal rat liver (Table 1). At 24 h post-partial hepatectomy SSAT activity in transgenic animals was immensely enhanced. Northern-blot analysis indicated that the stimulation of the enzyme activity was at least partly attributable to transcriptional activation (results not shown). The induction of SSAT was accompanied by a profound reduction of the hepatic spermidine pool, such that, at 24 h, liver spermidine concentration in the transgenic animals represented only 13% of that in their non-transgenic littermates (Table 1). The decrease in the spermidine pool in transgenic livers was transient, as pre-operative values were reached by 72 h post-operatively representing 40% of that in syngenic livers at that time point. Groups of syngenic and transgenic rats were also

treated with the polyamine analogue DENSPM, which is a powerful inducer of SSAT [15]. The modest enhancement of SSAT activity in syngenic animals was accompanied by a massive putrescine accumulation and total prevention of regeneration-induced increase in spermidine concentration at 24 h post-operatively, whereas these changes were no longer apparent at 72 h (Table 1). The drug dramatically induced SSAT activity in transgenic animals, especially at 24 h, and further decreased spermidine pools. While in syngenic animals ODC activity was only transiently stimulated, it remained at a high level in the transgenic animals throughout the whole period of observation. Multiple comparisons within the two groups indicated that DENSPM significantly increased putrescine ($P < 0.001$) and decreased spermidine ($P < 0.005$) at 24 h in syngenic animals. In transgenic animals the analogue significantly decreased spermidine ($P < 0.001$) at 72 h and further induced SSAT ($P < 0.001$) at 24 h. The constitutively high ODC activity in the transgenic livers probably contributed to the reappearance of spermidine at 72 h (Table 1).

Liver weight gain and proliferative activity were totally inhibited in transgenic rats during early liver regeneration

Figure 1(A) depicts the ultimate measurement of liver regeneration, i.e. the weight gain of the liver remnant. While in the syngenic animals liver weight was significantly ($P < 0.001$) increased at 24 and 72 h, the transgenic animals showed no weight gain until 72 h post-operatively, when the spermidine concentration reached the pre-operative level. Multiple comparisons revealed that liver weight gain was significantly ($P < 0.001$) inhibited in the transgenic animals at 24 h, but not at 72 h. Treatment of the animals with the polyamine analogue DENSPM slowed down the weight gain in both syngenic and transgenic animals (Figure 1A). Liver regeneration was followed for up to 8 days, revealing that, after 3 days, the liver weight gain was similar in both groups, and the hepatic spermidine concentrations in the transgenic animals, while never reaching the level found in syngenic regenerating livers, fluctuated between 40 and 60% of that found in non-transgenic animals at a given time point (results not shown). Not only was the weight gain similar in both groups after day 3, but the liver in both cases had a similar consistency and cellularity. This suggests that only half of the hepatic spermidine normally found after partial hepatectomy is sufficient to maintain normal liver regeneration.

PCNA was used as an indicator of cell proliferation during liver regeneration. Immunohistochemical detection of PCNA is a commonly used method to grade proliferative activity in a given tissue. PCNA expression is closely correlated with the S-phase of the cell cycle; it is superior to thymidine or 5-bromo-2-deoxyuridine incorporation because it does not involve any injections *in vivo* [16,17]. The number of PCNA-positive cells in resting liver, transgenic or not, was very low, around 1% (Figure 1B). Partial hepatectomy dramatically increased the PCNA labelling index (percentage of PCNA-positive cells of all liver cells) to about 20% in syngenic animals at 24 h ($P < 0.001$) and 72 h ($P < 0.001$) post-operatively, whereas transgenic animals did not show any proliferative activity at 24 h (labelling index less than 1%), and at 72 h the labelling index (5%) was significantly ($P < 0.001$) lower in comparison with the syngenic animals. The apparent discrepancy between the liver weight gain (Figure 1A) and PCNA index (Figure 1B) in the transgenic animals at 72 h post-operatively is in all likelihood related to the fact that the regeneration was initiated by water accumulation in the remnant, as normally occurs after partial hepatectomy,

Figure 3 Correlation between PCNA-positive cells and liver spermidine concentration at 24 h and 72 h after partial hepatectomy

Each point represents an individual animal not treated or treated with DENSPM obtained from the experiment depicted in Figure 1.

followed by proliferative activity. DENSPM significantly ($P < 0.001$) decreased the percentage of PCNA-positive cells in syngenic animals both at 24 and 72 h (Figure 1B).

In a separate experiment, thymidine incorporation into the acid-insoluble fraction of hepatic homogenates, before and at 24 h after partial hepatectomy, in both syngenic and transgenic animals was measured. In contrast with the syngenic animals (thymidine incorporation before the operation was 7060 ± 2100 and at 24 h post-operatively 45190 ± 24160 c.p.m./g of liver; $P < 0.05$), the transgenic animals showed no enhanced incorporation of thymidine at 24 h after partial hepatectomy (pre-operatively 7480 ± 610 and at 24 h post-operatively 6450 ± 560 c.p.m./g of liver; not significant). As in the previous experiment, hepatic spermidine decreased to 20% of the pre-operative level in the transgenic animals at 24 h after partial hepatectomy (results not shown). Figure 2 shows typical PCNA immunohistochemistry of liver sections obtained from syngenic (Figure 2A) and transgenic (Figure 2B) animals at 24 h after partial hepatectomy (samples from the thymidine incorporation experiment). As indicated in Figure 2, the difference between syngenic and transgenic animals was dramatic, as the transgenic livers did not show any proliferative activity whatsoever.

We also found a strikingly close and highly significant ($P < 0.001$) correlation between the PCNA labelling index and hepatic spermidine concentration during liver regeneration (among individual animals treated or not treated with the polyamine analogue) (Figure 3). Also, the hepatic concentration of spermine showed a positive correlation with the PCNA labelling index, while the concentration of putrescine negatively correlated with the apparent number of S-phase cells.

Unlike the total prevention of the liver weight gain, PCNA labelling index and thymidine incorporation in the transgenic animals at 24 h post-operatively, the incorporation of [14 C]leucine into total liver proteins was only marginally affected in the transgenic rats at this time point ($65 \pm 29\%$ versus $42 \pm 12\%$ increase in syngenic and transgenic animals, respectively; $P < 0.28$).

DISCUSSION

The role of increased accumulation of the polyamines (mainly putrescine and spermidine) during rat liver regeneration has remained as a matter of controversy. Inhibition of ODC by 2-difluoromethylornithine, an irreversible inhibitor of the enzyme, has been reported to retard rat liver regeneration [18]. On the other hand, prevention of the increases in ODC activity and putrescine, but not spermidine, accumulation did not have any effect on liver regeneration [19]. It is likely that pharmacological inhibition of polyamine biosynthesis does not result in a sufficiently massive reduction of the liver spermidine pool without overt toxicity. The results presented here strongly suggest that spermidine and/or spermine, but not putrescine, is specifically required for rat liver regeneration. Putrescine appears to serve as a precursor for spermidine, and spermine as a deposit to be used for the back-conversion into spermidine. The latter view is supported by the finding that transgenic animals maintained a normal spermidine pool apparently at the cost of spermine, which was decreased by 90% (Table 1). According to the results presented, a mere prevention of regeneration-induced increase in the hepatic spermidine pool may not be sufficient to prevent the regeneration process; the polyamine concentration must also decrease well below the pre-operative level. In fact, the regeneration started on day 3 and proceeded normally in transgenic livers at a spermidine level that was roughly half of that found in regenerating syngenic livers. It is highly likely that the constitutively high ODC activity in transgenic animals (Table 1) was able to supply sufficient amounts of spermidine for the maintenance of liver regeneration.

The process in which spermidine is required for rat liver regeneration is not known. Spermidine has a specific function to serve as a precursor for hypusine, an integral part of eukaryotic initiation factor 5A [20]. Inhibition of spermidine formation is known to lead to growth inhibition through hypusine depletion, but this is a slow process, where 50% reduction of hypusine level takes nearly a week [21] and hence is an unlikely mechanism during early liver regeneration. The latter view is also supported by the finding indicating that total protein synthesis was not significantly affected in the regenerating livers of transgenic rats.

The possible contribution of oxidative stress, resulting from the action of polyamine oxidase, could not be reliably excluded, as the specific inhibitor of polyamine oxidase, MDL72527 [8,22], turned out to be extremely toxic to partially hepatectomized transgenic rats, killing them within 24 h. The mechanism of this toxicity is not known, but we have found previously that, under conditions where SSAT is moderately induced, MDL72527 administration results in a striking superinduction of the enzyme [8].

We recently found that spermidine appears to be required for the integrity of the pancreas, as induction of SSAT by zinc, but not DENSPM, and the subsequent depletion of spermidine rapidly induces pancreatitis in these transgenic rats [8]. A possible contribution of pancreatic inflammation to the observed inhibition of liver regeneration was unlikely, as, apart from some pancreatic oedema being obvious in the partially hepatectomized transgenic rats, we did not find any signs of a real pancreatic inflammation, nor was serum α -amylase elevated in any of the groups of experimental animals.

The presented results strongly suggest that spermidine or spermine, but not putrescine, is critically involved either in the entry of cells into S-phase or in the progression of S-phase. This view is supported by the extremely close correlation found between hepatic spermidine level and the PCNA labelling index during early liver regeneration (Figure 3). Although a similar,

but smaller, correlation was also found between hepatic spermine and the PCNA labelling index, the kinetics of polyamine accumulation after partial hepatectomy, i.e. an early increase in spermidine and simultaneous decrease in spermine, seem to assign a critical role for spermidine during early rat liver regeneration. The results are also in excellent agreement with two other reports [23,24] revealing a strikingly close correlation between the number of cells in S-phase and the concentration of spermidine, but not that of putrescine or spermine, under conditions where polyamines were depleted as a result of a combined inhibition of their two biosynthetic decarboxylases.

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