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THYMIDYLATE SYNTHETASE AND THYMIDINE KINASE ACTIVITIES IN RAT GROWING CARTILAGE

BY

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ABSTRACT

The purpose of the present study is to determine whether or not the differences in cellular proliferation in rat nasal septal cartilage and tibial cartilage are reflected by differences in the pattern of age-related changes in thymidylate synthetase (TS) and thymidine kinase (TK) activities. TS and TK are responsible for thymidine monophosphate formation via, respectively, the *de novo* and the salvage pathways. The present study also examines the effects of glucocorticoids, which are known to affect the proliferation of chondrocytes, on TS and TK activities in these cartilaginous tissues.

Both enzyme activities declined with age, and the decline in TK activity was more rapid than that of TS. The TK/TS ratio decreased with age more rapidly in nasal septal cartilage. These findings suggest that nasal septal cartilage might mature earlier than tibial cartilage and, furthermore, that in rapidly growing tissues the salvage pathway functions predominantly.

Prednisone injection (2.5 mg/100 g, at 4 days after birth) clearly disturbed the gain of body weight. TK activity significantly decreased concomitantly with this arrest in weight gain, while in contrast TS activity was not depressed. This marked difference between TS and TK activities induced by prednisone suggests that these two enzymes may be regulated independently.

Key words: Thymidylate synthetase, Thymidine kinase, Nasal septal cartilage, Tibial cartilage, Growth.

INTRODUCTION

The role of nasal septal cartilage in craniofacial growth and development has been the focus of considerable attention and controversy; some investigators have suggested that nasal septal cartilage is a primary "direction-determining" factor in midfacial growth (Scott [1]), whereas others have asserted that it is a secondary growth site which merely keeps pace with the expansion of neighboring structures (Moss *et al.* [2]).

Previous attempts to resolve this question through experimental resection of nasal septal cartilage have yielded mixed results, depending on the age and species of experimental animal and the exact site and extent of resection (Ohyama [3]); Stenstrom and Thilander [4]; Sarnat and Wexler [5]). Subsequently, autoradiographic studies by Searls *et al.* [6–10] have demonstrated that thymidine uptake in nasal septal cartilage in rats declines rapidly at the end of gestation and almost vanishes during the early postnatal period. Based on these results, it was suggested that experimental removal of the nasal septal cartilage should be performed within ten days postpartum, in order to clarify its role in facial development.

In contrast to the early decline in cellu-

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Table 1. Experimental animals

	Age	No. of rats	No. of samples Septum	Tibia
Experiment 1	19 days of gestation	76	5	5
	1 day after birth	95	6	8
	4 days after birth	58	6	10
	7 days after birth	46	6	10
	10 days after birth	63	8	9
	14 days after birth	37	8	4
	28 days after birth	36	6	7
	Total	411	45	53
Experiment 2	Prednisone: 4 days after birth	23	8	8
	Control: 4 days after birth	22	8	8
	Total	45		

lar proliferation observed in rat nasal septal cartilage, the cartilaginous growth plates of long bones, such as the tibia, continue to undergo cellular proliferation even after puberty. This suggests that differential mechanisms may be involved in the control of chondrocyte proliferation in these two tissues.

DNA-synthesizing enzyme activity is a significant factor in the regulation of cellular growth and proliferation; thus, it seems likely that tissues which seem to have differing mechanisms for the control of cellular proliferation, such as the nasal septal and tibial cartilage, may also exhibit differences in the pattern of age-related changes in DNA-synthesizing enzyme activities.

The purpose of the present study is to determine whether or not the differences in chondrocyte proliferation in rat nasal septal cartilage and tibial epiphyseal cartilage are in fact reflected by differences in the temporal pattern of changes in activities of the DNA-synthesizing enzymes, thymidylate synthetase (TS; EC 2.1.1.45) and thymidine kinase (TK; EC 2.7.1.21), which are responsible for, respectively, the *de novo* and salvage synthesis of thymidine monophosphate (TMP). Furthermore, since it is known that glucocorticoids may seriously impair cartilage growth, the present study also examines the effects of glucocorticoid administration and subsequent growth inhibition on TS and TK activities in nasal septal and tibial cartilage.

Materials and Methods

I. *Experimental animals*

Male and female Wistar strain rats were employed throughout the experiments (Table 1). They were kept under controlled environmental conditions (room temperature: 25°C, relative humidity: 55–65% and lights on between 08:00 and 20:00). Standard pellets and tap water *ad libitum* were supplied. Litters were housed with the mothers up to 21 days postpartum. Beyond 21 days after birth, they were housed in group cages (5–6 rats/cage).

A. Experiment 1: Age-related changes in TS and TK activities

Four hundred and eleven rats in seven different age groups ranging from 19 days of gestation to 28 days after birth were used.

B. Experiment 2: Effect of prednisone injection on enzyme activities

Forty-five, 4-day-old rats, were used. Twenty-three rats were subcutaneously injected with prednisone (2.5 mg/100 g, Sigma), a synthetic corticosteroid, in 0.2m*l* of 0.9% NaCl solution 18 hours before sacrifice. Twenty-two control rats were treated in the same manner with 0.9% NaCl alone.

II. *Preparation of enzyme extract*

Nasal septal cartilage and proximal tibial cartilaginous area were removed and surrounding fibrous tissues were carefully stripped off in ice-cold 0.9% NaCl solution. They were weighed and stored at −80°C. About 50 mg of frozen tissue was considered as one unit of sample. They were pulverized, suspended in 10 volumes of 0.15 M KCl and homogenized in a glass homogenizer with a Teflon pestle for 20 strokes. The homogenate was centrifuged at 25,000×g for 20 minutes. The supernatant was used for enzyme assays. The protein concentration of the enzyme extract was determined by the method of Lowry *et al.* [11] with bovine serum albumin as a reference standard.

III. *Assay of TS activity*

TS activity was assayed according to the method of Dunlap *et al.* [12]. Fifty microliters of enzyme extract was mixed with 300 μl of reaction mixture containing final concentrations of 71.4 mM potassium phosphate (pH 6.8), 17.9 mM NaF, 3.6 mM $NaHCO_3$, 5 mM HCHO, 17.9 mM 2-mercaptoethanol, 0.14 mM tetrahydrofolate and 67.4 nM [5-^{3}H] deoxyuridine monophosphate ([5-^{3}H]dUMP, 10.6 Ci/mM, Amersham). After the mixture was incubated at 37°C for 10 minutes, the reaction was terminated by the addition of 50μl of ice-cold 10% trichloroacetic acid, and then 100 μl of a charcoal suspension (80 mg/m*l*) was added to absorb unreacted [5-^{3}H]dUMP. The mixture was centrifuged, and 100 μl of the supernatant was counted in 5 m*l* of a Triton/toluene-based scintillation fluid. TS activity was expressed as pmol TMP formed per minute per mg protein.

IV. *Assay of TK activity*

TK activity was determined by the method of Taylor *et al.* [13]. The reaction mixture contained 40 μl of the enzyme preparation combined with 160 μl of assay solution to give final concentrations of 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 10 mM ATP and 1 μM [6-^{3}H] thymidine (20 Ci/mM, Amersham). The mixture was incubated at 37°C for 15 minutes and the reaction was stopped by boiling for 3 minutes. After centrifugation, 100 μl aliquots of the mixture were spotted on 1.8-cm squares of DEAE-cellulose paper (DE81, Whatman). The dried squares were put in 1 mM ammonium formate solution and washed four times. Finally the squares were rinsed in 99% ethanol and dried at 70°C. The radioactivity of the dried square was counted in 10 m*l* of a toluene-based scintillation fluid. TK activity was expressed as pmol TMP formed per minute per mg protein.

Results

I. Experiment 1: Age-related changes in TS and TK activities

The age-related changes in TS activity are shown in Fig. 1. In the nasal septal cartilage, TS activity during the first postnatal week showed no significant differences from the level of activity found in the fetal stage, and then it declined within the next week. On the other hand, in the proximal tibial cartilage, the activity significantly decreased from the fetal level at one day after birth, and thereafter it showed almost the same pattern of changes as seen in the nasal septal cartilage.

The age-related changes in TK activity are shown in Fig. 2. TK activities in both tissues decreased rapidly from the high

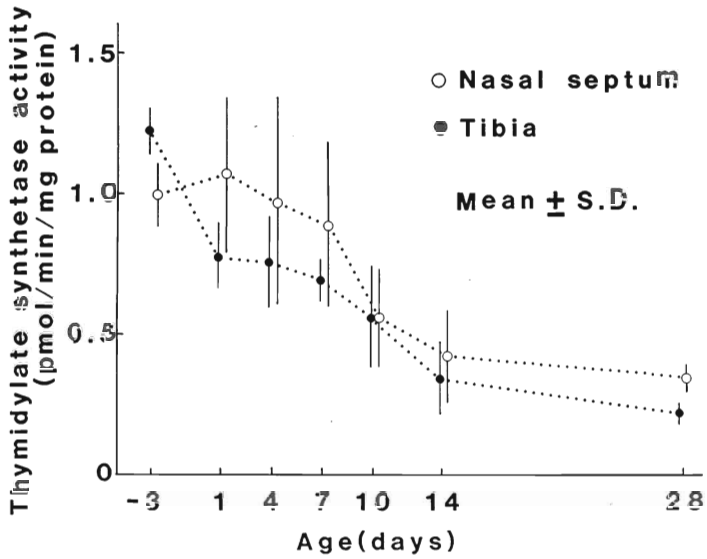

Fig. 1. Age-related changes in TS activity in rat nasal septal and tibial cartilage

TS activity was assayed according to the method of Dunlap *et al.* [12] and was expressed as pmol TMP formed per min per mg protein.

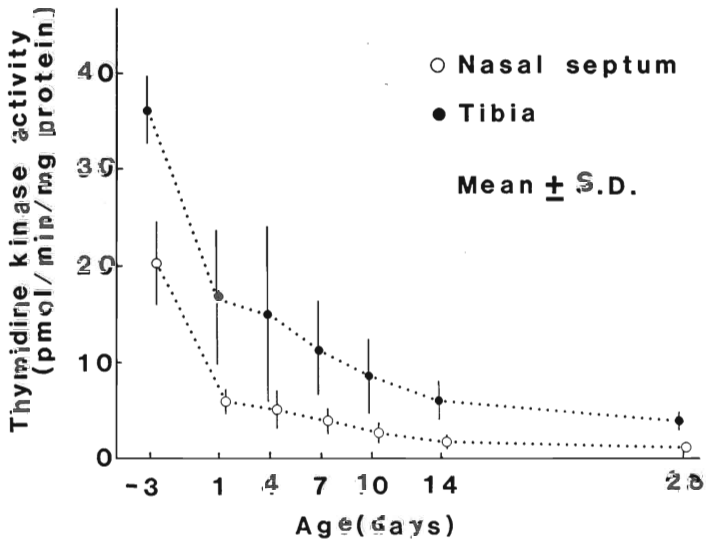

Fig. 2. Age-related changes in TK activity in rat nasal septal and tibial cartilage

TK activity was determined by the method of Taylor *et al.* [13] and was expressed as pmol TMP formed per min per mg protein.

fetal level at one day after birth with subsequent gradual decline. It was also found that TK in the tibial cartilage showed a higher activity than that of the nasal septal cartilage at each stage.

The age-related changes of TK/TS ratio were calculated and plotted in Fig. 3. The ratio in the nasal septal cartilage showed a

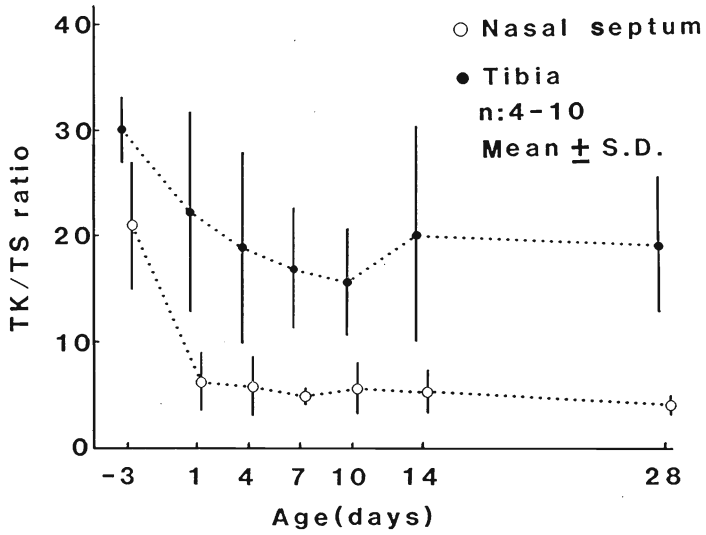

Fig. 3. Age-related changes of TK/TS ratio in rat nasal septal and tibial cartilage

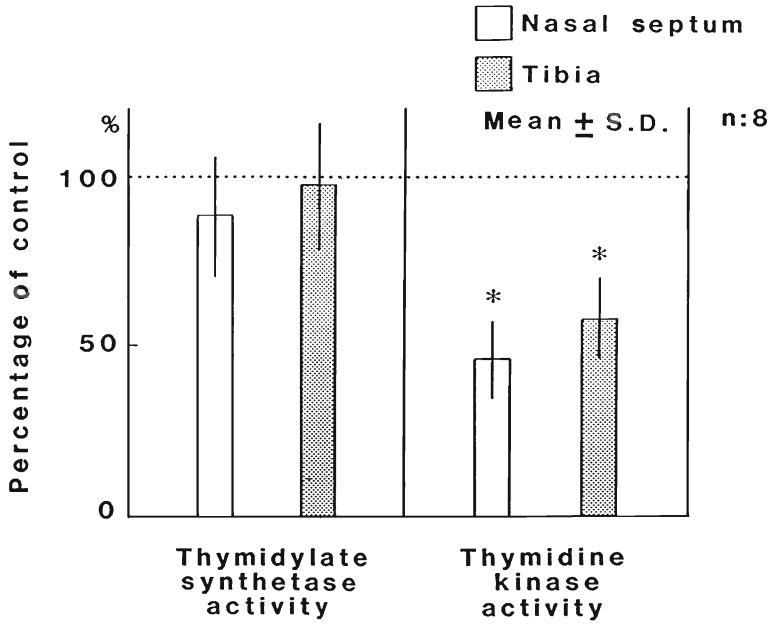

Fig. 4. Effect of prednisone injection on TS and TK activities in rat nasal septal and tibial cartilage

Four-day-old rats were subcutaneously injected with prednisone (2.5 mg/100 g) in 0.2 m*l* of 0.9% NaCl solution or with 0.9% NaCl alone 18 h before sacrifice. TS and TK activity measurements of prednisone-treated animals were divided by those of control and the calculated percentages are shown.

*Significant difference compared to control value. ($P<0.01$)

Table 2. Effect of prednisone on body weight

	Before treatment (4-day-old)	18h after (5-day-old)
Control (n: 22)	11.97±1.04	13.17±1.22
Prednisone (n: 23)	11.91±0.92	*11.98±0.91

Mean body weight(g)±S.D.
* Significant difference compared to matched control ($p<0.01$).

significant difference between the prenatal and the postnatal stage. The latter maintained a considerably low value. On the other hand, the ratio in the tibial cartilage decreased gradually with age, but about 60% of the fetal value still remained at 28 days.

II. Experiment 2: Effect of prednisone injection on enzyme activities

Percentage activities of TS and TK in prednisone-treated animals relative to those of control are shown in Fig. 4. In the prednisone-treated group, the body weight ceased to increase and the mean weight was significantly less than that of the control group (Table 2).

TS activity did not differ significantly between the prednisone-treated and the control groups. On the other hand, TK activities in the septal and the tibial cartilage showed 54.1% and 41.8% decrease, respectively.

Discussion

In the present study, the author investigated TS and TK activities in cartilage obtained from the nasal septum and proximal tibia. Cellular proliferation of nasal septal cartilage decreases at the early postnatal stage, whereas the cartilaginous growth plate of the tibia continues to undergo cellular proliferation after puberty. There have been no previous studies related to TS and TK activities in the growing cartilage of these tissues. Both enzymes play important roles in the biosynthesis of TMP, an essential and specific precursor of DNA. TS is the only enzyme which can catalyze *de novo* TMP formation and has aroused the interest of many biochemists as a target in cancer chemotherapy (Friedkin [14]). TK catalyzes the phosphorylation of thymidine via the pyrimidine salvage pathway, and high levels of TK activity have been reported in several rapidly proliferating tissues such as fetal tissues (Machovich and Greengard [15]; Herzfeld *et al.* [16]), regenerating liver (Nawata and Kamiya [17]), tumor tissue (Sakamoto *et al.* [18]) and estrogen-stimulated uterus (Sakamoto *et al.* [19]).

As shown in Figs. 1 and 2, both TS and TK activities were at high levels in the fetal cartilage and declined after birth. This decline was more rapid in TK activity than in TS activity in both nasal septal and tibial cartilage. High levels of TS and TK activities in fetus with subsequent decline after birth was in agreement with the series of autoradiographic studies on nasal septal cartilaginous growth using tritiated thymidine by Searls *et al.* [6–10]. They demonstrated that the thymidine uptake was high between 16 and 19 days of gestation and drastically decreased before birth and stayed at low level thereafter. Their findings and the present results suggest that cartilaginous growth activity in the nasal septum is at high levels in the fetal stage.

In this study, the TK/TS ratio is shown in Fig. 3. The author found significant differences in age-related changes of the TK/TS ratio between the nasal septal cartilage and the tibial cartilage. These differences may be due to the different patterns of change in TS activity occurring one day after birth, and also may be due to the significantly lower TK activity in nasal septal cartilage throughout all stages. In view of these results, it is interesting to

note that Herzfeld and Raper [20] reported that the TK/TS ratios in several internal organs decreased with maturation. Thus, the present results suggest that nasal septal cartilage might mature earlier than tibial cartilage. Furthermore, the TK/TS ratio difference found in both tissues suggests that, in rapidly growing tissues, the salvage pathway functions predominantly.

Glucocorticoids are well known to affect skeletal growth, and their effects on cartilage have been widely investigated (Silbermann [21]; Takano *et al.* [22]). One possible mechanism for such effects is suggested by the fact that glucocorticoids are known to affect the DNA-synthesizing enzymes, TS and TK. In hypophysectomized rat adipose tissue, the stimulation of TK by growth hormone was prevented by cortisone (Epstein *et al.* [23]). In addition, hydrocortisone decreases the level of TK (Greengard and Machovich [24]). TK activity has been reported to be more sensitive to hydrocortisone than TS activity (Herzfeld and Raper [20]). In the present study, effect of prednisone, a synthetic glucocorticoid, on TS and TK activities in nasal septal and tibial cartilage was examined. The prednisone-treated group showed a significant decrease of increment of body weight within only 18 hours (Table 2). It was interesting to find that TK activity decreased concomitantly with this arrest in weight gain, while in contrast TS activity was not depressed in spite of such a drastic growth inhibition. Marked difference between TS and TK activities induced by prednisone, which is coincident with the findings of Herzfeld and Raper [20], suggests that these two enzymes may be regulated independently.

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