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Comparison of Sulfate Metabolism in Costal Cartilage of Normal and 'Little' Mice

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Abstract

C57BL/6J and mutant 'little' (*lit*/*lit*) mice *c.* 50 days of age were injected with doses of [^{35}S]sulfate proportional to their body weight. Despite the diminished growth rate of *lit*/*lit* mice compared with normal mice at this age, uptake of radioactivity per unit mass of cartilage was similar for both mouse types, confirming previous data. Additional experiments with these mice established that the similarity of sulfate uptake could not be accounted for by differences in the location of bound sulfate or (for females) by differences in cartilage cellularity. Investigation of sulfate loss by costal cartilage *in vivo* indicated that cartilage degradation occurred at a greater rate in *lit*/*lit* mice than in normally growing mice. These latter data suggest that growth hormone, which is lacking in *lit*/*lit* mice, may in part regulate skeletal growth (at least for female mice) by inhibiting degradation of cartilage.

Introduction

The mechanism by which growth hormone exerts its growth-promoting effects upon skeletal cartilage has not been established, although it is widely held that somatomedins (Daughaday *et al.* 1972) play a central role.

Although considerable data is available to support this concept (see review by Phillips and Vassilipoulou-Sellin 1980), the demonstration that serum factors defined as somatomedins possess the ability to stimulate *in vivo* growth is limited to three recent reports (van Buul-Offers and Van Den Brande 1979; Holder *et al.* 1981; Schoenle *et al.* 1982). The first two studies showed that repeated injections of serum fractions enriched in somatomedin activity stimulated growth of Snell dwarf mice, while the work of Schoenle *et al.* (1982) established that the growth factor known as IGF 1 stimulates growth of hypophysectomized rats.

The above data strongly support the somatomedin concept, in contrast to results obtained by McKern *et al.* (1981) in studies with 'little' (*lit*/*lit*) mice. These mice have been proposed as animal models for isolated growth-hormone deficiency (Eicher and Beamer 1976; Beamer and Eicher 1976), although there is evidence to suggest that there may also be a deficiency of prolactin in males and pregnant females (Keough and Wood 1979). On the other hand, virgin *lit*/*lit* mice show no evidence of a functional deficiency of prolactin (Beamer and Eicher 1976; Keough and Wood 1979; Phillips *et al.* 1982).

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The studies of McKern *et al.* (1981) showed that uptake of injected radioactive sulfate per gram of costal cartilage of *lit/lit* mice *in vivo* was similar to that of C57BL/6J mice. Because circulating levels of somatomedin in the former were considerably reduced compared with the latter (Nissley *et al.* 1980; McKern *et al.* 1981), the similarity of cartilage sulfation by the two types of mouse suggested that somatomedin was not a primary regulator of cartilage synthesis *in vivo*.

The results of this paper confirm and extend the above findings, and demonstrate that sulfate loss from costal cartilage of *lit/lit* mice is significantly greater than that of C57BL/6J mice.

Materials and Methods

Animals

C57BL/6J mice, heterozygous *lit/+* mice and mutant *lit/lit* mice (derived from breeding pairs supplied by the Jackson Laboratory, Bar Harbor, Maine) were maintained as previously described (McKern *et al.* 1981). Unless otherwise stated, injections were given intraperitoneally as solutions in 0·9% (w/v) saline. Blood was collected via the subclavian artery during ether anaesthesia, centrifuged at 5°C and the serum stored at −20°C.

Uptake of [^{35}S]Sulfate

The mice listed in Table 1 were each injected with $3{\cdot}7 \times 10^{10}$ Bq per gram body weight of carrier-free [^{35}S]sodium sulfate (obtained from The Radiochemical Centre, Amersham, England) After 24 h the amount of radioactivity bound by cartilages 6 and 7 was determined as before (McKern *et al.* 1981). In some of the mice, costal cartilages 4 and 5 were also removed, cleaned, air-dried and weighed, then digested for 18 h at 60°C in 0·5 ml of a 0·1% (w/v) solution of twice-recrystallized papain (Sigma Chemical Company), buffered at pH 5·6 with 0·1 M potassium phosphate containing 20 mM EDTA and 20 mM cysteine hydrochloride. Following centrifugation, supernatants were adjusted to pH 7·4 with 0·1 M NaOH, diluted twofold with 0·1 M Tris buffer of the same pH, and loaded onto columns 2 cm long by 1 cm diameter of DEAE cellulose (DE-52, Whatman Ltd, Maidstone, Kent), pre-equilibrated with the buffer. After copious washing of the columns with this buffer, glycosaminoglycans were eluted by applying the same buffer containing 2 M KCl. Aliquots (0·5 ml) from fractions were mixed with 7 ml of Dimilume-30 (Packard Instrument Company, Illinois) and radioactivity determined on the ^{14}C channel of a Packard Tri-Carb liquid scintillation spectrophotometer.

Determination of DNA

The concentration of DNA was determined in costal cartilages 6 and 7 from C57BL/6J and *lit/lit* mice approximately 70 days of age using the method of Kissane and Robins (1958), modified as described by Graystone (1979). This procedure utilizes the reaction between aminobenzoic acid and the deoxyribose sugars produced following acid hydrolysis of DNA at 60°C.

Loss of [^{35}]Sulfate from Mouse Cartilage

Loss of bound [^{35}S]sulfate was measured by injecting mice with a body-weight-related dose of radioactive sulfate as described before. The mice were kept for 21–22 days before being killed. Radioactivity in costal cartilages was then determined as for sulfate-uptake experiments.

Isolation of Total Skeletal Cartilage

In some experiments, the amount of [^{35}S]sulfate bound by the total amount of skeletal cartilage was determined by first injecting mice with a dose of radioactive sulfate approximately 10 times that injected into mice where costal cartilage was excised, to enable the accurate measurement of [^{35}S]sulfate content in subsequent aliquots of dissolved skeleton. Mice were killed at designated times and skinned, eviscerated carcases exposed to a colony of *Dermestes maculatus* beetles and their larvae. Because of their dietary preference for dry protein, these insects have been used to pro-

duce clean skeletons from rats and other small mammals (Hefti *et al.* 1980). Careful monitoring of the cleaning process enabled the production of mouse skeletons with intact cartilaginous components within 1 or 2 days (McKern 1982).

Statistical Analysis

Student's *t*-test was used to compare data from normal mice in each experiment with that from *lit/lit* mice in the same experiment.

Results

Sulfate Uptake by Costal Cartilage

Uptake of [^{35}S]sulfate *in vivo* by costal cartilage of *lit/lit* mice was not significantly different (at the $P<0{\cdot}05$ level) from that of costal cartilage from C57BL/6J or *lit/+* mice (Table 1), in any of six separate experiments. Inclusion or deletion of the

Table 1. Comparison of sulfate uptake *in vivo* by cartilage of C57BL/6J, *lit/+* and *lit/lit* mice

Mouse type	No. and sex of mice	Age (days)	Mean [^{35}S]sulfate uptake±s.d. (dpm/mg dry wt)[A]	Student's *t*-test values[B]
Experiment 1				
C57BL/6J	6 females	47–48	1675±528	0·78
lit/lit	6 females	47–48	1492±237	
Experiment 2				
C57BL/6J	6 females	47–48	1278±342	0·27
lit/lit	6 females	47–48	1235±198	
Experiment 3				
C57BL/6J	6 females	44–45	1323±382	1·51
lit/+	3 males, 4 females	44–45	1105±368	
lit/lit	6 males, 3 females	44–45	1055±258	0·31
Experiment 4				
lit/+	7 males, 3 females	46–48	2158±1195	0·30
lit/lit	7 males, 3 females	46–48	2280±398	
Experiment 5				
lit/+	5 males, 4 females	46–58	1315±247	0·14
lit/lit	7 males, 5 females	48–52	1335±412	
Experiment 6				
lit/+	7 males, 1 female	46–49	1307±425	0·64
lit/lit	4 males, 3 females	46–47	1188±250	

[A] All mice were injected with 37 kBq [^{35}S]sodium sulfate per gram body weight. Radioactivity was measured 24 h later in costal cartilages 4–7 (experiments 1–3) or 6–7 (experiments 4–6), including the osteochondral junction in all cases except experiment 2. Solutions were injected intravenously via the orbital plexus in experiment 3, but intraperitoneally in all other experiments.

[B] Data for normal mice in each experiment was compared with that from *lit/lit* mice in the same experiment. In all cases the value for *t* indicated that the difference was not significant at the $P<0{\cdot}05$ level.

osteochondral junction did not appear to influence this result (experiment 2 compared with the other experiments), nor did changing the injection mode from intraperitoneal to intravenous (experiment 3 compared with the other experiments).

Location of [^{35}S]*Sulfate in Costal Cartilage of* lit/+ *and* lit/lit *Mice*

The concentration of [^{35}S]sulfate in aliquots from papain digests of costal cartilages 4 and 5 from the mice used in Experiment 6 (Table 1) was measured. Mean values (dpm/mg)±s.d. for digest solutions and DE-52 column fractions were:

	Papain digest	0·1 M KCl eluate	2·0 M KCl eluate
lit/+	419±143	27±14	448± 80
lit/*lit*	351± 60	37±12	419±137

It can be seen that similar amounts of radioactivity were present in solutions from *lit*/+ and *lit*/*lit* cartilage and that most of the radioactivity was bound to DEAE-cellulose at pH 7·4 in 0·1 M KCl in both mouse groups, but was eluted by buffer containing 2·0 M KCl. These results suggest that radioactivity was largely bound to glycosaminoglycan molecules of costal cartilage (Handley and Lowther 1976) of *lit*/*lit* and *lit*/+ mice.

DNA Concentration in Costal Cartilage

The mean (±s.d.) concentration of DNA in costal cartilage of six female C57BL/6J mice, 70 days old, was 0·485±0·028 μg per milligram dry weight, which was not significantly different from the concentration of 0·487±0·101 μg/mg for six female *lit*/*lit* mice. By contrast, the concentration of DNA in costal cartilage from six male C57BL/6J mice 50 days old was 0·336±0·058 μg/mg dry weight, significantly less ($P<0·05$) than the concentration of 0·491±0·135 μg/mg in costal cartilage from six male *lit*/*lit* mice.

Table 2. Body weight changes and specific growth rates of C57BL/6J mice and *lit*/*lit* mice of similar age

Means±s.d. are shown

Parameter	C57BL/6J Mice	*lit*/*lit* Mice
Number and sex	7 females, 2 males	6 females, 2 males
Ages on day 0 (days)	43–46	44–45
Body weight (*g*)		
Day 0	16·5±2·3	9·9±0·5
Day 10	19·4±2·4	10·8±0·5
Difference	2·9±1·2	0·9±0·3[A]
10^3 × Specific growth rate per day	17·8±7·3	9·2±2·8[B]

[A] $P<0·001$ compared with C57BL/6J mice.
[B] $P<0·01$ compared with C57BL/6J mice.

Increase in Body Weight of Normal and 'Little' Mice

During the period averaging from 45 to 55 days of age, weight gain in a group of C57BL/6J mice was significantly greater than that for a group of *lit*/*lit* mice of similar age (Table 2). Since initial body weights of the C57BL/6J mice were considerably greater than those of the *lit*/*lit* mice, it is more appropriate to compare the fractional increase in initial body weight per unit time (the specific growth rate) rather than absolute weight gain. Mean specific growth rate of the C57BL/6J mice was considerably greater than that of the *lit*/*lit* mice (Table 2). Similar observations were

made when body weight changes in *lit*/+ and *lit*/*lit* mice were compared during the period from 40 to 64 days of age (data not shown), consistent with results of previous comparisons of these genotypes (Beamer and Eicher 1976; Graystone 1979).

Sulfate Loss from Costal Cartilage of lit/+ *and* lit/lit *Mice*

The mean amount ±s.d. of radioactivity retained *in vivo* by costal cartilage from seven *lit*/*lit* mice, 21 days after injection of [^{35}S]sulfate, was 439±102 dpm/mg which was 28% less than the 615±106 dpm/mg retained by cartilage from *lit*/+ mice ($P<0{\cdot}05$). In a second experiment, the mean amount ±s.d. of radioactivity retained by costal cartilage from *lit*/*lit* mice was 537±78 dpm/mg, 22% less than the 684±253 dpm/mg measured in cartilage from *lit*/+ mice, 22 days after injection of [^{35}S]sulfate. In this experiment, although the difference in cartilage radioactivity was not significant, the small amount of radioactivity contained in the 5 mm of bone adjacent to the osteochondral junction was significantly less ($P<0{\cdot}05$) in *lit*/*lit* mice (22±21 dpm/mg) compared with that in *lit*/+ mice (66±25 dpm/mg).

Uptake and Loss of [^{35}S]*Sulfate by Whole Skeletons*

Mean (±s.d.) radioactivity per gram dry weight of skeleton from four *lit*/+ mice and six *lit*/*lit* mice, killed 22 h after injection of [^{35}S]sulfate, was 75 403±21 990 dpm and 88 044±22 377 dpm respectively, the difference being not significant at the $P<0{\cdot}05$ level. By contrast, when whole skeletons were obtained from nine C57BL/6J and eight *lit*/*lit* mice 18 days after injection with [^{35}S]sulfate, mean (±s.d.) radioactivity per gram was 28 228±5 860 dpm and 12 282±2 046 dpm respectively ($P<0{\cdot}001$).

Discussion

Results of the present study have confirmed those obtained previously (McKern *et al.* 1981) which showed that uptake of radiosulfate by costal cartilage *in vivo* was similar for mutant *lit*/*lit* and phenotypically normal *lit*/+ mice (Table 1). In addition, it has been demonstrated that sulfate uptake by costal cartilage of C57BL/6J mice is similar to that of *lit*/+ and *lit*/*lit* mice of similar age (experiments 1–3). Variation of experimental conditions such as the mode of injection of the radioactive bolus, or deletion of the osteochondral junction of the cartilage segment (Table 1), indicates that neither of these factors significantly alters the similarity of sulfate uptake by the different mouse types.

The results of Table 2 show that at the age used in this study, *lit*/*lit* mice have a much lower mean growth rate than C57BL/6J and *lit*/+ mice, consistent with data obtained by Beamer and Eicher (1976) and Graystone (1979). The similarity of sulfate uptake between mutant *lit*/*lit* and normal mice cannot therefore be ascribed to similar rates of growth. Since the relationship of skeletal weight to body weight has been shown to be similar for *lit*/*lit* and C57BL/6J mice (McKern 1982), it is likely that the rate of skeletal growth differs substantially between the two mouse types, at a time when costal cartilage synthesis (measured as sulfate incorporation) is similar.

This conclusion may be invalidated if sulfate incorporation by cartilage of *lit*/*lit* mice is grossly different to that by cartilage of unaffected mice. However, this does not appear to be the case, since most of the radioactivity bound to both *lit*/+ and *lit*/*lit* mouse cartilage was incorporated into the glycosaminoglycan fraction of digested

cartilage. Hence it is likely that sulfation of the chondroitin polysaccharide chains is similar in both mouse types (in contrast to results obtained with other abnormally growing mutants, such as the brachymorphic mouse—Orkin *et al.* 1976).

Sulfate is incorporated into proteoglycan via an intracellular process, therefore the comparison of sulfate uptake per unit mass of cartilage may be invalidated if the degree of cellularity of this tissue differs between the mice. However, since the results show that the cellularity of costal cartilages (DNA content per milligram of tissue) from female C57BL/6J mice was nearly identical to that from *lit/lit* females, measurement of radioactivity per unit mass is a valid method for comparing sulfate uptake in these animals. By contrast, for males the DNA content per milligram of cartilage from C57BL/6J mice was an average of 46% less than that of cartilage from *lit/lit* mice. It is not known whether this latter difference in the males is of sufficient magnitude to significantly alter the conclusion that sulfate incorporation is similar in normal and 'little' mice but since females were used exclusively in two of the experiments (Table 1, experiments 1 and 2), the hypothesis remains valid for the latter.

The finding that mean sulfate-uptake by whole skeletons of *lit/lit* mice is not significantly different from that of *lit/+* mice indicates that skeletal-cartilage synthesis as a whole may be similar in these mouse types. The data are consistent with the findings for sulfate uptake by costal cartilage and suggest that the rate of synthesis of skeletal cartilage may not correlate with growth rate in these mice.

Studies of cartilage proteoglycans (using [^{35}S]sulfate as the labelled precursor) indicate that there is considerable heterogeneity in turnover rate between different types of cartilage and different species (Maroudas 1974). Bostrom (1952) estimated that for normal adult rats the half-life of [^{35}S]sulfate retention was 17 days. The results of studies with NMRI mice by Herbai (1970) suggested the presence of 'resting' costal cartilage distal to the sternum and 'active' costal cartilage proximal to, and including, the osteochondral junction. The half-life of [^{35}S]sulfate retention was about 30 days for the former and 8 days for the latter. Based on these data, measurements of radioactivity in mouse costal cartilage within 24 h of injection of [^{35}S]sulfate are essentially reflecting cartilage synthesis (Table 1), while those made 18–22 days after injection of radioisotope are likely to be predominantly reflecting rates of cartilage degradation.

Results of the present study therefore indicate that costal cartilage in *lit/lit* mice is degrading at a faster rate than that in normal mice although a major time-course study with considerable numbers of mice is required to confirm this point. The observation that sulfate loss from the whole skeleton is considerably greater in *lit/lit* mice compared with normal mice suggests that the metabolism of all skeletal cartilage in the former is on average less than that in the latter. The wide variation in individual results and small sample size necessitates caution, however, when interpreting the data. Despite such limitations, the data offer a logical explanation for the observed similarity in cartilage synthesis (as measured by sulfate uptake) between *lit/lit* and normal mice, since greater degradation of cartilage tissue in the former would result in the observed decreased growth rate compared with unaffected mice.

It can be postulated that the greater degradation observed with *lit/lit* mice may be a consequence of the deficiency of growth hormone (although the possible additional deficiency of prolactin in males causes some difficulty in assessing hormonal effects in that sex). Hence, growth hormone may regulate skeletal growth in mice predom-

inantly by suppressing degradation. A consequence of this postulate is that, although somatomedin activity (Daughaday *et al.* 1972) may play a role in regulating processes of skeletal growth by stimulating cartilage synthesis, the dominant effect may be one of inhibition of cartilage degradation. In this regard, it is of interest to note the finding by Ballard *et al.* (1980) that a number of purified growth factors, including some that qualify as somatomedins, markedly inhibited protein degradation at physiological concentration in a number of cell lines. There may be factors in serum which are intermediaries of growth hormone and which act by inhibiting degradation of cartilage tissue, but these may not be detectable by *in vitro* assays which measure sulfate uptake.

Supporting evidence that increased degradation rather than decreased synthesis is at least partly responsible for the diminished growth of *lit/lit* mice comes from studies of their skeletal muscle growth (Graystone 1979). Protein synthetic rates were shown to be equivalent in skeletal muscle of *lit/lit* and *lit/+* mice. From this and other data it was concluded that protein degradation was increased in *lit/lit* mice, since they had subnormal amounts of muscle protein.

From the results of the present studies it may be concluded that measurement of the net effect of cartilage synthesis and degradation is a necessary prerequisite for meaningful studies of mechanisms controlling skeletal growth (at least in the mice used in these studies). In this context, it may not always be sufficient to equate the level of serum somatomedin in an animal model as an index of its growth potential.

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