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Endocrine Control of Cartilage Growth in Coho Salmon: GH Influence *in Vivo* on the Response to IGF-I *in Vitro*

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ABSTRACT—Ceratobranchial cartilages from coho salmon (*Oncorhynchus kisutch*) parr, injected with growth hormone (GH) at 4μg/g body weight or with saline, were sampled monthly from February to July. Thymidine and sulfate uptakes by cartilages were determined as measures of DNA and chondroitin sulfate synthesis, respectively. Cartilages were incubated with IGF-I at 0.01, 0.1 and 1μg/ml to examine the *in vitro* response to this hormone. GH injection increased cartilage thymidine and sulfate uptakes at least four-fold in all experiments. IGF-I treatment *in vitro* further increased sulfate but not thymidine uptake in cartilages from GH-injected coho and increased uptake of both in cartilages from saline-treated coho. However, the IGF-stimulated uptakes were still significantly below the uptakes in cartilages from GH-injected coho. The dual effector hypothesis of GH action [12] in mammals is supported at least in part in teleost fishes by the observation that addition of IGF-I *in vitro* was not equivalent to injection of GH *in vivo*.

INTRODUCTION

The endocrine control of cartilage growth has only recently been examined in teleost fish [see 1, 2, 20]. Studies on the Japanese eel, *Anguilla japonica*, by Duan and Inui [8, 9] have shown that the stimulatory action of GH on sulfate uptake by cartilage is indirect. Duan and Hirano [6, 7] later showed that sulfate uptake by eel cartilage is stimulated by mammalian IGF-I and raised the possibility of regulation by a similar principle in teleosts. McCormick *et al.* [14] and Gray and Kelley [10] have subsequently shown that mammalian IGF-I stimulated sulfate incorporation *in vitro* in cartilages from coho salmon (*O. kisutch*) and goby (*Gillichthys mirabilis*), respectively. These observations are consistent with the somatomedin hypothesis [4].

In anadromous salmonids, smoltification is a period during which the fish undergoes various physiological changes, many of which are cued by the endocrine system. Endogenous levels of growth hormone, prolactin, thyroid hormones and cortisol change in a distinctive pattern in coho salmon (*Oncorhynchus kisutch*) undergoing smoltification [19]. In the period when GH levels in the coho are increasing [16, 19], cartilage growth rate would be expected to increase due to increased liver-derived IGF-I in the circulation [3, 18] and to sensitization of the cartilage to IGF-I by GH [12]. Although injection of GH leads to transient down-regulation of liver GH receptors [10, 11, 15, 17] in several teleost species, increased expression of IGF-I mRNA in the liver was observed in coho salmon [5]. Injection of GH, comparable to natural increases in endogenous GH, may thus stimulate cartilage growth.

The purpose of this study is to examine further the effect of GH on cartilage growth and its potential interaction with IGF-I. *In vivo* GH action and *in vitro* IGF-I action on ceratobranchial cartilage in coho salmon were judged by determining thymidine and sulfate incorporation. Experiments were done repeatedly during the period of parr-smolt transformation to detect possible developmental or seasonal changes in response to GH *in vivo* and to IGF-I *in vitro*.

MATERIALS AND METHODS

Animals

Coho salmon (*Oncorhynchus kisutch*) parr (10–20 g) were obtained from Iron Gate Hatchery, California Department of Fish and Game, in December 1991. They were maintained outdoors at Bodega Marine Laboratory at 12–14°C in a 2000-liter concrete raceway supplied with filtered fresh water and were fed twice daily with Oregon Moist Pellets (Moore-Clarke, LaConner, WA) at a ration of 2% body wt/day.

Injections

The fish for each monthly experiment were randomly separated into two groups: GH-injected (NIADDK-oGH-15 at 4μg/g body wt; n=10) and saline-injected (n=10). The oGH was dissolved in 0.01 N NaOH followed by saline solution to yield a final concentration of 2μg/μl solution (with pH less than 9); the same volume of 0.01 N NaOH was added to the saline used for injecting controls. Fish were injected with 2μl solution/g body wt on alternate days (total of 4 injections).

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Sampling

Cartilage samples were taken monthly from the above groups from February to July. Fish remained unfed for 7 days before sampling in an attempt to increase the sensitivity of their cartilage to IGF-I [13]. Fish were killed by a blow to the head 24 hr after the last injection. Ceratobranchial cartilages were dissected from the bone of the first three pairs of gill arches of each fish under a dissecting microscope and placed in a pre-culture medium: Minimum Essential Medium (MEM) with Hanks' salts, penicillin (100 U/ml) and streptomycin (100μg/ml). Randomly-selected cartilages (with an average dry wt of 53$\pm$15.6μg in February to 101$\pm$27.2μg in July) from each fish were then placed into wells (24-well plate, Falcon 3047) for different treatments (n=7–10 for each treatment): basal (untreated); non-specific (cartilages frozen at -80°C to measure non-specific thymidine and sulfate uptake); recombinant bovine IGF-I (rbIGF-I; a gift from Monsanto, St. Louis, MO, U.S.A.) at 0.01, 0.1 and 1μg/ml. Each well contained 1 ml culture medium: MEM with Earle's salts, bovine serum albumin (BSA; 25μg/ml), penicillin (50 U/ml), streptomycin (50μg/ml), $^{35}SO_4$ (1μCi/ml) and ^{3}H-thymidine (2μCi/ml). The cartilages were then incubated in a chamber filled with 95% O_2/5% CO_2 at 14°C for 48 hr. During this period, the ceratobranchial cartilage incorporated radioactive sulfate into chondroitin sulfate and radioactive thymidine into DNA. The experiment was terminated by freezing at -80°C. Cartilages were then soaked in cold Na_2SO_4 twice and rinsed with distilled water 3 times in order to eliminate residual unincorporated radioactive sulfate and thymidine. The cartilages were dried in an oven at 60°C and weighed to the nearest μg. Each cartilage was then placed in a scintillation vial containing 0.5 ml 99% formic acid which dissolved the cartilage, thereby releasing the radioactivity ($^{35}SO_4$+^{3}H-thymidine) into the acid. Liquid scintillation fluid (4.5 ml) was added to each vial. ^{35}S and ^{3}H radioactivities in dpm were determined by a dual-label (^{3}H and ^{35}S) program in a Beckman 5000 scintillation counter. The dpm count was normalized for each cartilage weight to yield dpm/μg.

Statistical analysis

Two-way analysis of variance (ANOVA) was used to test for significance of GH injection over time. All other statistical comparisons were done by one-way ANOVA followed by Newman-Keuls analysis for post-hoc comparisons of factor means. Regression analyses and ANOVA were conducted using the Crisp statistical program (CRUNCH, Berkeley, CA). All groups comprised 7–10 fish, and $P \leq 0.05$ was considered significant.

RESULTS

Body weight and smoltification

Mean body weight increased linearly (with a slight decrease in June) from 18 g in February to 39 g in July (data not shown). Signs of smoltification (loss of parr marks, silvering of scales, darkening at edge of fins, and increased condition factor) were most evident in May.

Thymidine and sulfate uptakes

Thymidine uptake (Fig. 1) by the cartilages in GH-injected coho was 4 to 16 times higher than in saline-injected coho throughout the study period (two-way ANOVA, $P < 0.0001$). From February to June, levels of thymidine uptake

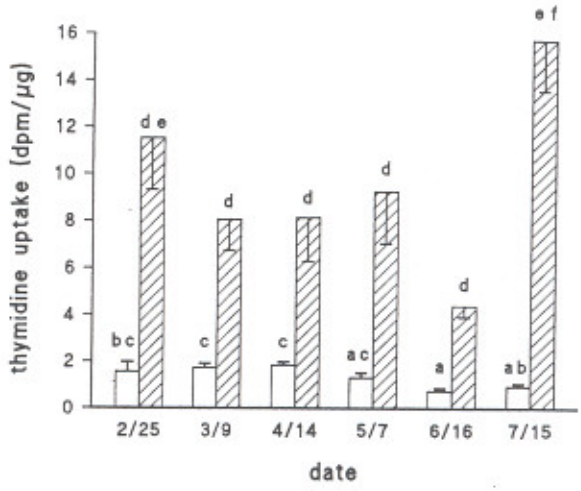

FIG. 1. Basal thymidine uptake (dpm/μg cartilage) by ceratobranchial cartilages from GH- and saline-injected coho salmon (*Oncorhynchus kisutch*) sampled monthly from February to July. Fish were given injections on alternate days (total of 4 injections) and sampled 1 day after the last injection. Hatched and clear boxes represent GH- and saline-injected fish, respectively. Values are mean$\pm$SEM (n=7–10). Values with shared letters are not significantly different ($P > 0.05$).

by cartilage from GH-injected coho showed a decreasing trend without statistical significance, averaging 10 dpm/μg from February to May and dropping to 4.4 dpm/μg in June. Thymidine uptake increased to 15.7 dpm/μg in July ($P < 0.05$ compared to the uptake in March-June). Thymidine uptake in saline-injected coho averaged 1.6 dpm/μg from February to May, and dropped ($P < 0.05$) to 0.8 dpm/μg in June and July.

Sulfate uptake (Fig. 2) by the cartilages in GH-injected

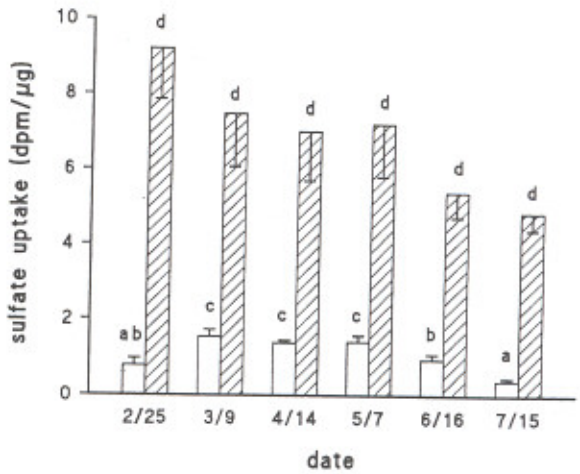

FIG. 2. Basal sulfate uptake (dpm/μg cartilage) by ceratobranchial cartilages from GH- and saline-injected coho salmon (*Oncorhynchus kisutch*) sampled monthly from February to July. Fish were given injections on alternate days (total of 4 injections) and sampled 1 day after the last injection. Hatched and clear boxes represent GH- and saline-injected fish, respectively. Values are mean$\pm$SEM (n=7–10). Values with shared letters are not significantly different ($P > 0.05$).

TABLE 1. Monthly measurements from February-July of thymidine uptake (dpm/μg) by cartilages from GH- and saline-injected (SAL) coho in response to IGF-I *in vitro*

	IGF-I (μg/ml)			
	0	0.01	0.1	1
Feb.				
GH	11.5±2.2 (8)	11.1±1.6 (8)	17.2±2.9 (9)	18.7±2.0 (9)
SAL	1.5±0.4 (8)	2.1±0.4 (9)	2.6±0.3 (9)	3.2±0.4 (10)*
Mar.				
GH	8.1±1.3 (9)	6.7±1.8 (8)	8.5±1.8 (9)	7.3±1.0 (9)
SAL	1.7±0.2 (10)	1.5±0.2 (8)	1.9±0.2 (10)	2.6±0.2 (10)*
Apr.				
GH	8.2±1.9 (9)	6.6±1.1 (8)	11.3±1.8 (9)	11.3±1.8 (9)
SAL	1.8±0.2 (8)	2.1±0.2 (9)	2.9±0.3 (7)*	3.0±0.2 (8)*
May				
GH	9.3±2.2 (9)	11.8±2.2 (10)	22.7±4.7 (10)*	16.9±3.4 (10)
SAL	1.3±0.2 (9)	2.4±0.3 (10)*	3.0±0.2 (10)*	2.9±0.3 (10)*
June				
GH	4.4±0.5 (7)	4.8±0.5 (8)	6.2±0.5 (8)	6.2±0.6 (9)
SAL	0.7±0.1 (8)	1.9±0.3 (8)*	1.9±0.3 (8)*	2.1±0.2 (8)*
July				
GH	15.8±2.2 (7)	21.8±2.8 (7)	21.2±4.3 (7)	18.3±2.3 (7)
SAL	0.9±0.1 (7)	1.9±0.4 (7)*	2.3±0.4 (7)*	2.2±0.3 (7)*

data expressed as ^{3}H-thymidine dpm/μg±SEM (N)
* $P<0.05$ over basal (0) uptake

TABLE 2. Monthly measurements from February-July of sulfate uptake (dpm/μg) by cartilages from GH- and saline-injected (SAL) coho in response to IGF-I *in vitro*

	IGF-I (μg/ml)			
	0	0.01	0.1	1
Feb.				
GH	9.2±1.4 (8)	9.5±0.9 (9)	14.4±1.8 (9)*	14.9±1.0 (9)*
SAL	0.8±0.2 (8)	1.8±0.3 (9)*	2.7±0.3 (9)*	3.1±0.2 (10)*
Mar.				
GH	7.5±1.4 (9)	6.3±1.2 (8)	8.2±1.4 (9)	8.2±1.3 (9)
SAL	1.5±0.2 (10)	1.4±0.2 (10)	1.9±0.2 (10)	2.4±0.3 (10)*
Apr.				
GH	7.0±1.3 (9)	6.3±0.8 (8)	10.8±1.2 (9)*	10.5±1.2 (9)*
SAL	1.4±0.1 (8)	1.5±0.1 (9)	2.7±0.2 (7)*	2.6±0.2 (8)*
May				
GH	7.2±1.4 (9)	11.1±1.6 (10)	17.5±2.8 (10)*	14.1±2.0 (10)*
SAL	1.4±0.2 (9)	2.8±0.3 (10)*	3.4±0.2 (10)*	3.3±0.3 (10)*
June				
GH	5.4±0.7 (7)	6.1±0.5 (8)	7.1±0.5 (8)	7.7±0.9 (9)*
SAL	0.9±0.1 (8)	2.4±0.4 (8)*	2.2±0.3 (8)*	2.2±0.2 (8)*
July				
GH	4.9±0.5 (7)	7.8±0.7 (7)*	6.4±0.8 (7)	6.7±0.7 (7)
SAL	0.4±0.1 (7)	1.0±0.2 (7)*	1.2±0.2 (7)*	1.1±0.2 (7)*

data expressed as $^{35}SO_4$ dpm/μg±SEM (N)
* $P<0.05$ over basal (0) uptake

coho was 5 to 11 times higher than in saline-injected coho throughout the study period (two-way ANOVA, $P<0.0001$). Sulfate uptake by cartilages in GH-injected coho showed a decreasing trend without statistical significance, averaging 8 dpm/μg from February to May and dropping to 5 dpm/μg in June and July. Uptake in saline-injected coho was 0.8 dpm/μg in February, then increased ($P<0.05$) to an average of 1.4 dpm/μg from March to May, and dropped ($P<0.05$) to 0.9 and 0.4 dpm/μg in June and July, respectively. Thymidine and sulfate uptakes were positively correlated in both GH-treated fish ($r=0.65$, $P<0.001$) and in saline-treated fish ($r=0.68$, $P<0.001$).

Cartilage response to IGF-I in vitro

Cartilages from GH- and saline-treated fish in each month were tested for their response to IGF-I *in vitro*; the results are presented in Table 1 (thymidine uptake) and Table 2 (sulfate uptake).

In thymidine uptake (see Table 1), cartilages from GH-treated fish did not respond significantly to further stimulation by IGF-I *in vitro* at 0.01, 0.1 and 1μg/ml; an exception was in May, when cartilage treated with IGF-I *in vitro* at 0.1μg/ml showed an increase over the basal uptake ($P<0.05$). Cartilages from the saline-treated group responded to *in vitro* IGF-I at 1μg/ml in February and March ($P<0.05$), then to both 0.1 and 1μg/ml IGF-I in April ($P<0.05$), and to all IGF-I doses from May to July ($P<0.05$). However, cartilages did not show a dose-dependent response to IGF-I over the doses tested: uptake generally plateaued with increasing doses of IGF-I after the initial or smallest dose that elicited a response.

In sulfate uptake (see Table 2), cartilages from GH-treated fish responded to IGF-I *in vitro* with increases in all months except March. Cartilages sampled in February, April and May responded to IGF-I *in vitro* at 0.1 and 1μg/ml ($P<0.05$). A dose-dependent response to IGF-I was not found, as stimulated sulfate uptake plateaued after 0.1μg/ml. Cartilages in June and July only responded to 1μg/ml and 0.01μg/ml IGF-I, respectively. Cartilages from the saline-treated group responded to *in vitro* IGF-I in all months. Cartilages sampled in February, May, June and July all responded to *in vitro* IGF-I at 0.01, 0.1 and 1μg/ml. Cartilages in March only showed stimulated sulfate uptake at 1μg/ml IGF-I, whereas cartilages in April responded to IGF-I at 0.1 and 1μg/ml. A dose-dependent response to IGF-I again was not found, as stimulated sulfate uptake usually plateaued after the initial or smallest dose that elicited a response.

DISCUSSION

As seen in Figures 1 and 2, GH injection markedly increased thymidine and sulfate uptakes, and the two parameters are strongly correlated ($r=0.65$, $P<0.001$). This indicates that thymidine and sulfate uptakes are generally coupled, even after GH stimulation.

Cartilages from saline-injected fish showed decreased thymidine uptake (Fig. 1) and decreased sulfate uptake (Fig. 2) in June and July. A marked decline in Na^+, K^+-ATPase activity (an indicator of hypoosmoregulatory activity) also occurred in these fish in June and July [13], which may indicate the end of the smoltification period. Cartilages from GH-injected fish also showed a non-significant trend of decreasing thymidine (Fig. 1) and sulfate uptakes (Fig. 2) from February to June and from February to July, respectively. Injected GH might have compensated for the expected decrease in endogenous GH (plasma GH levels were not measured), resulting in a lack of significant decrease in both thymidine and sulfate uptakes. A significant increase in GH-stimulated thymidine uptake ($P<0.05$) was, however, observed in July. This is contrary to the sulfate uptake which stayed low. One explanation may be that the cartilages undergo a new cycle of chondrocyte proliferation at this time. A major increase in thymidine uptake indicating mitotic activity in prechondrocytes/chondrocytes resulted from GH injection in July; this may have led to increased sulfate uptake by maturing chondrocytes at a later date, but this was not examined.

Although GH injection consistently increased thymidine and sulfate uptakes, it did not result in a consistent sensitization to IGF-I *in vitro* as judged by thymidine uptake. The dual effector hypothesis [12] predicts that GH would increase serum levels of IGF-I and increase cartilage sensitivity to IGF-I. Thus, injected GH may have maximally stimulated the mitotic activity of chondrocytes *in vivo* so that further stimulation by IGF-I *in vitro* was not seen. This is in contrast to the findings which showed that priming of gill Na^+, K^+-ATPase resulted from either endogenous or exogenous GH, so that further stimulation by IGF-I *in vitro* was possible [13]. As chondrocytes can also respond to IGF-I by synthesizing chondroitin sulfate, further stimulation of sulfate uptake by IGF-I *in vitro* was still possible. Thus, consistent IGF-I stimulation of sulfate uptake was observed in cartilages from GH-injected coho (March was the only exception).

Although cartilages from the saline-injected group consistently responded to IGF-I *in vitro* with stimulated thymidine and sulfate uptakes, the stimulated uptakes did not approach the basal uptake seen in the GH-injected group (Tables 1 and 2). The observation that GH *in vitro* at 1μg/ml did not increase thymidine or sulfate uptake [14; Tsai, unpublished] suggested that GH has no direct effect on cartilage growth. GH injection may thus act by increasing endogenous IGF-I levels and/or by sensitizing the cartilage cells to hepatic and/or local IGF-I. The dual effector hypothesis of GH action [12] is supported by the observation that no dose of IGF-I alone *in vitro* was able to parallel the effects of GH injection *in vivo*. However, the organ-culture system used did not allow testing of IGF-I *in vitro* in the presence of other serum factors, including IGF-binding proteins. Such factors may modify the responsiveness of cartilage to stimulation by IGF-I. Furthermore, the exposure time of cartilages to IGF-I *in vitro* for 48 hr (maximal sulfate incorporation by eel and salmon cartilage occurs between 24

and 48 hr [8, 9, 14]) was significantly less than the exposure *in vivo* to GH, which was given as 4 injections during a 9-day period (these fish were the same as those used by Madsen and Bern [12]).

As the cartilages seemed to have responded maximally to GH injection and also to IGF-I addition *in vitro*, no significant seasonal change could be discerned. These studies support the relevance of the dual effector theory of GH action [12] to teleost cartilage growth: injection of GH *in vivo* induced consistently higher thymidine and sulfate uptakes by cartilage than were seen in the control cartilages exposed to IGF-I *in vitro*.

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