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Effects of Human Carbonic Anhydrase III (CA III) on Synovial and Muscle Fibroblast Glycosaminoglycan Metabolism (42441)

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Abstract. We investigated the ability of CA III, isolated from adult human skeletal muscle, to regulate cell growth and glycosaminoglycan (GAG) formation in connective tissue cells derived from various human tissues. Unlike muscle, dermal, and cartilage fibroblasts, synovial connective tissue cells were substantially activated by CA III and showed enhanced hyaluronic acid (HA) synthesis. Cell culture experiments showed that CA III induced a 2- to 11-fold increase in [^{14}C]HA synthesis by human synovial fibroblasts (SF) in a dose-dependent manner ($P < 0.001$); erythrocyte CA I and CA II were inactive. Exposure of SF and muscle fibroblasts to CA III also resulted in a 20–45% and 16–70% increased ^{35}S incorporation into proteoglycans, respectively. When adult human skin and cartilage fibroblasts were studied in the presence of CA III, no differences in the level of DNA and GAG formation were noted. These latter cell types were clearly activated by a platelet (CTAP-III) growth factor. The potential physiological implications of these observations are discussed.

Carbonic anhydrase (CA) (EC 4.2.1.1.) catalyzes the reversible hydration of carbon dioxide; three distinct isoenzymes (CA I, CA II, CA III) have been characterized. The amino acid sequences of human CA I and CA II are known (1), and that of human CA III has been recently determined (2). CA III was first discovered in mammalian red skeletal muscle and subsequent studies have shown it to be present in male rat liver, and, at much lower levels, in rabbit liver, sheep lung, and in human smooth and cardiac muscle (1). More recently, low concentrations of CA III have also been found in human red blood cells (3). Kinetic and inhibition studies indicate that CA III is a less efficient CO_2 hydratase than CA I and CA II, and that it is only weakly inhibited by sulfonamides. This variation in activity and in distribution raises several interesting questions, including the possibility that CA III may have nonenzymatic function(s) (1).

In the course of identifying, isolating and characterizing a human spleen derived growth factor (4), we utilized the responsiveness of human synovial cells in bioassay systems to guide protein fractionation. CA I was identified as one protein component present in biologically active preparations. Consequently, experiments were designed to ascertain whether purified CA I and its homologous CA II and CA III counterparts had the ability to stimulate extracellular matrix formation and/or DNA synthesis in appropriate target cell cultures. This report, which has appeared in abstract form (5, 6), describes that CA III induced a 2- to 11-fold increase in [^{14}C]HA synthesis by human synovial fibroblasts; erythrocyte CA I and CA II were inactive.

Materials and Methods. Normal human synovial, dermal, and cartilage cells were isolated by methods previously described (7). In brief, fibroblast monolayer cultures were developed from explants obtained at arthrotomy or amputation and grown in CMRL 1066 (GIBCO, Grand Island, N.Y.) medium containing 15% fetal calf serum (FCS, Reheis Chemical Co., Kankakee, Ill.), 5% heat-inactivated normal human serum, penicillin (Pfizer, New York, N.Y.), streptomycin (Pfizer), L-glutamine (Sigma, St. Louis, Mo.), 0.02 *M* Hepes (Sigma), and sodium bicarbonate. Complete medium changes were carried out three times a week and trypsin (Sigma) dispersal was performed as required for propagation or study. Normal muscle fibroblasts were propagated from fresh postmortem human psoas major muscle explants as described above. Cells used in this study had been transferred five to seven times.

Protein purification. Human CA III was purified from red skeletal muscle obtained at autopsy as previously reported (8). Crude extracts

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were dialyzed overnight against 5 m*M* Tris–SO_4, 1 m*M* 2-mercaptoethanol, pH 8.7, and then passed twice through a Prontosil-CM Sephadex (Pharmacia Fine Chemicals, Uppsala, Sweden) column (2.5 × 40 cm) which had been equilibrated with the dialysis buffer. Fractions were monitored for CA activity, determined by a bromothymol blue spot test, pooled, dialyzed, vacuum concentrated, and further purified by passage through a Sephadex G-75 column (3 × 110 cm, Pharmacia) equilibrated with 2 m*M* Tris–HCl and 1 m*M* 2-mercaptoethanol, pH 8.0. Fractions containing CA III were identified as before and examined for purity by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (9). Homogenous CA III fractions were pooled, vacuum concentrated, dialyzed overnight at 4°C against approximately 200 vol of physiologic saline, and filtered–sterilized (0.45 μ*M;* Millipore, Bedford, Mass.) in preparation for bioassay. Connective tissue activating peptide III (CTAP-III) was isolated from outdated normal human platelets as described (10).

Tryptic digestion. This was done as previously described (8). In brief, approximately 2.7 mg of purified human CA III was denatured by lowering the pH to 2.0 with 1 *N* HCl and then raising the pH to 11.0 with 1 *N* NaOH. The pH was then readjusted to 8.5 with concentrated HCl. A 1/10 vol of 0.5 *M* Tris–HCl pH 8.6 was added to buffer the digestion. Trypsin (Worthington Biochemicals Corp., Freehold, N.J.) was added (1/50 w/w) and the reaction was allowed to proceed for 16 hr at 37°C. As controls similar amounts of CA III accompanied the above experiments but the denaturation and trypsinization aspects were omitted. Samples were then filtered–sterilized for bioassay. The efficiency of the method has been previously described (8).

Isotope incorporation studies. As reported (11), 2×10^4 cells were plated in microtiter wells (Costar) nourished with assay medium (100 μl/well) consisting of Eagle's synthetic medium (ESM, GIBCO) and supplemented with 3% FCS, antibiotics, L-glutamine, Hepes, and Na_2CO_3. After 20–24 hr incubation at 37°C in a humidified chamber to allow cell attachment and spreading, the medim was supplemented with another 100 μl of assay medium containing 0.5 μCi/ml of uniformly labeled [D-^{14}C]glucosamine–HCl ([^{14}C]Gm) or 50 μCi/ml of $^{35}SO_4$ (sp act 647.54 mCi/mmol, New England Nuclear, Boston, Mass.). Twenty microliters of the agonist or its vehicle was then added at the reported concentrations. These microcultures (four wells/group) were then incubated for an additional 40–48 hr after which time the assay was terminated by spotting 100 μl of medium on Whatman 3MM chromatography paper. Radiolabeled GAG was fixed to the paper with a 5-min exposure to 0.1% cetylpyridinium chloride (CPC, Sigma); unincorporated label was removed by eight sequential washes, 5 min each, with 0.1 *M* NaOH for the assessment of [^{14}C]glucosamine-labeled GAG. When [$^{35}SO_4$] was used to label S-GAG, four washes, 30 min each, were employed using 0.1% CPC in 0.3 *M* NaCl. After the final wash, samples were dried at 35°C and counted in a Beckman LS-7000 scintillation counter using a toluene–POPOP–POP system.

Tritiated thymidine incorporation into fibroblast DNA. Cells were plated, 10^4 cells/well (four wells/group) in 100 μl of assay medium as described (9), and incubated in a humidified chamber at 35–37°C for 20-24 hr. Test agonists or vehicles were then added (10 μl/well) and incubation was resumed for 24 hr, at which time 15 μl of [^{3}H]methylthymidine (sp act 78.1 Ci/mmol, New England Nuclear) was added (1.5 μCi/well) and incubation resumed for an additional 24 hr. Medium was aspirated, discarded, and the cell sheets were washed twice, each separately, with phosphate-buffered saline, 5% TCA (BioRad Laboratories, Richmond, Ca.) and absolute methanol. After air drying, cells were lysed for 1 hr at 37°C with 0.3 *N* NaOH and then the lysate was spotted on glass paper, dried, and counted.

Enzymatic analysis of GAGs. Qualitative identification of GAGs was accomplished by incubating labeled media with hyaluronidase (ex. *Streptomyces hyalurolyticus,* Calbiochem) in 0.05 *M* sodium acetate, pH 5, chondroitinase ABC or AC (Miles Laboratory, Elkhart, Ind.) in 0.2 *M* Tris, pH 7.5, or the appropriate buffer for 3 hr at 37°C. The digested and control samples were subjected to the CPC fixation-wash procedure and GAG identity was determined on the basis of specific enzyme lability (11).

Statistical analysis. Statistical analysis was performed using the Student–Fisher *t*-test. Data are displayed as means ± 1 SD.

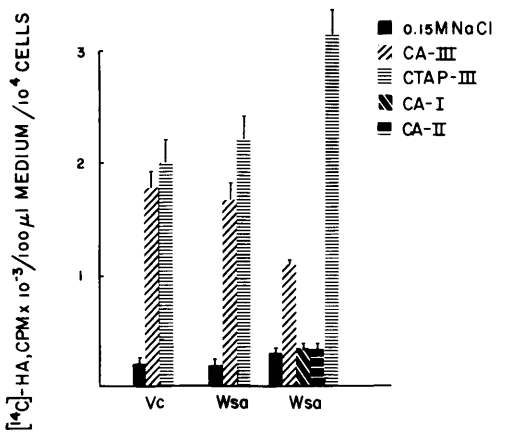

FIG. 1. Hyaluronic acid synthesis in human synovial cells in response to CA III (2×10^{-6} *M*) or CTAP-III (4×10^{-6} *M*). Vc and Wsa represent the initials of the donor. Wsa cell strain was studied in two different assays.

Results. *Hyaluronic acid (HA) formation.* In preliminary experiments we measured HA synthesis by human synovial cells in the presence or absence of CA III. As depicted in Fig. 1, synovial fibroblasts synthesized approximately 11 times more [^{14}C]Gm-labeled macromolecules in the presence of the agonist compared to saline-treated cultures ($P < 0.001$). On the other hand, the addition of similar concentrations of CA I or CA II (Sigma) resulted in baseline HA formation. One should note that the same CA III and

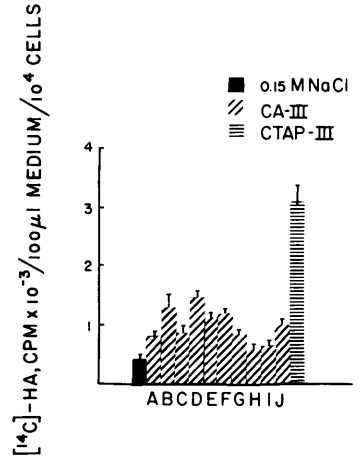

FIG. 2. Hyaluronic acid synthesis in human synovial cultures in response to constant concentrations (1.6×10^{-5} *M*) of 10 individual CA III samples. CTAP-III (4×10^{-6} *M*).

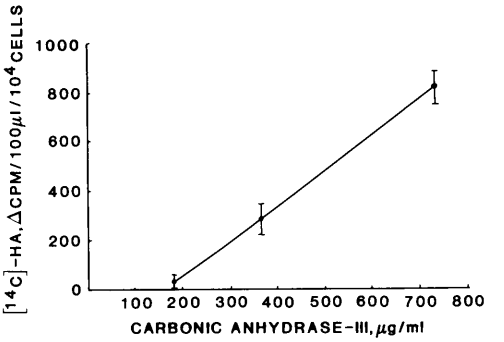

FIG. 3. Hyaluronic acid synthesis in human synovial cultures in response to increasing CA III concentrations (6×10^{-6} to 2.5×10^{-5} *M*).

CTAP-III preparations were assayed against the same synovial cell strain (Wsa) at different times; in both cases stimulation of HA metabolism by CA III and CTAP-III was seen. The degree of response varied, emphasizing the importance, when comparing substances, of analyzing them in the same experiment (10). We therefore simultaneously measured the GAG stimulatory activity of different CA III samples; Fig. 2 shows the results of this experiment. As can be seen, normal human synovial fibroblasts synthesized and secreted two- to fourfold more [^{14}C]Gm-labeled macromolecules, chiefly HA, in the presence of maximal concentrations (500 µg/ml) of 10 different CA III preparations ($P < 0.001$). As shown in Fig. 3, the addition of increasing amounts of highly purified CA III resulted in increasing HA formation in a dose-dependent fashion. Interestingly, as can be seen in Table I, complete tryptic digestion of the agonist (8) had no bearing on the stimulatory effects of CA III in enhancing HA synthesis by synovial fibroblast cultures.

TABLE I. TRYPSIN-TREATED CA III EFFECTS ON HYALURONIC ACID FORMATION

Additive	[^{14}C]HA/10^4 synovial cells	E/C[a]
0.15 *M* NaCl + trypsin	454 ± 34[b]	—
CA III + 0.15 *M* NaCl	916 ± 22	2.0[c]
CA-III + trypsin	1160 ± 64	2.6[c]

[a] E/C, experimental/control.
[b] Numbers represent cpm/100 µl of medium, expressed as means ± SD of four cultures.
[c] $P = <0.001$.

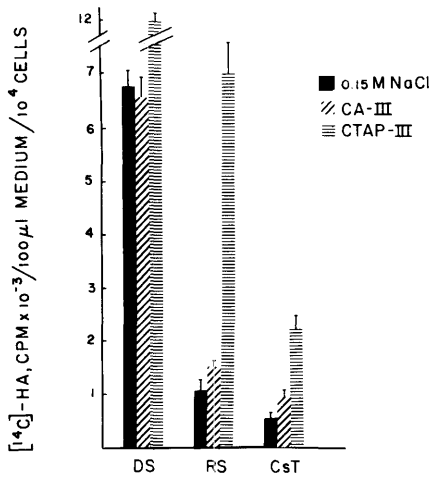

FIG. 4. Hyaluronic acid synthesis by three different human skeletal muscle fibroblast strains in response to CA III (1.3×10^{-5} *M*) or CTAP-III (4×10^{-6} *M*).

The effects of CA III on muscle fibroblast formation of HA were also studied. Unexpectedly (Fig. 4), muscle fibroblasts did not exhibit increased synthesis of HA formation in response to the same CA III concentrations (500 µg/ml) that stimulated SF. In the mediator-treated cultures, very high concentrations were used in an attempt to promote a maximal response. To examine further the spectrum of the GAG stimulatory effect on connective tissue cells isolated from various human tissues, synovial, muscle, skin, and cartilage, were tested simultaneously for incremental HA synthesis in the presence of CA III. As shown in Table II, CA III again stimulated HA formation in SF ($P < 0.001$), whereas dermal, muscle, and cartilage fibroblasts were relatively unresponsive to the HA-enhancing activity of the mediator ($P < 0.1$). Figure 4 also shows that CTAP-III stimulated HA synthesis two- to sixfold in these cultures.

Sulfated-GAG (S-GAG) and DNA synthesis. Synovial, muscle, skin, and cartilage fibroblast cultures were also tested for their DNA and proteoglycan biosynthetic profile in the presence or absence of human CA III. As shown in Tables II and III, a slight but statistically significant increase in S-GAG secreted by synovial and muscle fibroblasts was seen in CA III-treated cultures. In agreement with published data (12), CTAP-III induced two to three times more S-GAG synthesis in synovium- and cartilage-derived connective tissue cells. Human synovial, dermal, and muscle cells were also studied with respect to their capacity to synthesize [^{3}H]DNA in the presence or absence of CA III isoenzyme. As can be seen in Table IV, CA III caused 44% stimulation of DNA formation in synovial fibroblasts, whereas muscle and skin fibroblasts were unresponsive to the mitogenic actions of the agonist. Again, CTAP-III was mitogenic for all types of cells studied (11).

Characterization of GAG. Radiolabeled macromolecules released into the medium by synovial and muscle fibroblasts were characterized on the basis of culture medium hydro-

TABLE II. GLYCOSAMINOGLYCAN SYNTHESIS IN SYNOVIAL, MUSCLE, SKIN, AND CARTILAGE FIBROBLASTS

Additive	Synovial cells	E/C[a]	Muscle cells	E/C[a]	Skin cells	E/C[a]	Chondrocytes	E/C[a]
			[^{14}C]GAG					
0.15 *M* NaCl	212 ± 31[b]	—	1677 ± 111	—	2122 ± 287	—	4370 ± 836	—
CA III[c]	436 ± 30	2.0[d]	1454 ± 100	0.9	2281 ± 268	1.07	5051 ± 671	1.2
CTAP-III[e]	4169 ± 404	19[d]	11744 ± 1186	7.0[d]	12548 ± 921	6.0[d]	23691 ± 2806	5.4[d]
			[^{35}S]GAG					
0.15 *M* NaCl	1937 ± 224[b]	—	2417 ± 620	—	1665 ± 203	—	2756 ± 552	—
CA III[c]	2205 ± 230	1.13	2450 ± 369	1.0	1737 ± 173	1.0	2741 ± 258	1.0
CTAP-III[e]	5332 ± 353	2.80[d]	5864 ± 1108	2.4[d]	2378 ± 214	1.4[d]	10027 ± 1044	3.6[d]

[a] E/C, experimental/controls.
[b] Numbers represent cpm/100 µl of medium, expressed as means ± SD of six cultures.
[c] 1.2×10^{-5} *M*.
[d] $P = <0.001$.
[e] 10^{-6} *M*.

TABLE III. *S*-GLYCOSAMINOGLYCAN FORMATION IN SYNOVIAL AND MUSCLE FIBROBLAST CULTURES

Additive	Synovial strain	[^{35}S]GAG/ 10^4 cells	E/C[a]	Muscle strain	[^{35}S]GAG/ 10^4 cells	E/C[a]
0.15 *M* NaCl	PW	1774 ± 196[b]	—	DS	3318 ± 252	—
CA III[c]	PW	2169 ± 153	1.2[d]	DS	3849 ± 189	1.2[d]
CTAP-III[e]	PW	5113 ± 593	2.9[d]	DS	4161 ± 546	1.3[d]
0.15 *M* NaCl	AB	2796 ± 219	—	RS	390 ± 53	—
CA III[c]	AB	3760 ± 307	1.3[d]	RS	659 ± 75	1.7[d]
CTAP-III[e]	AB	6498 ± 894	2.3[d]	RS	1239 ± 206	3.2[d]
0.15 *M* NaCl	JM	2408 ± 314	—	CsT	1276 ± 51	—
CA III[c]	JM	3482 ± 217	1.4[d]	CsT	1971 ± 113	1.5[d]
CTAP-III[e]	JM	5970 ± 459	2.5[d]	CsT	2584 ± 288	2.0[d]

[a] E/C, experimental/control.
[b] Numbers represent cpm/100 μl of medium, expressed as means ± SD of six cultures.
[c] 1.6×10^{-5} *M*.
[d] $P = <0.001$.
[e] 10^{-6} *M*.

lysis by fungal hyaluronidase and chondroitinases AC and ABC. Table V shows that most of the [^{14}C]Gm-labeled GAG synthesized by both groups of cells was hyaluronic acid. When $^{35}SO_4$ was used as precursor, 60–70% was incorporated into chondroitin 4/6 sulfate, as determined by sensitivity to chondroitinases. Virtually no dermatan sulfate was detected, although we made no attempt to characterize cell-associated GAG. Of note was overall similarity in the kind of GAG molecules secreted by activated or nonactivated muscle and synovial fibroblast cultures.

Discussion. In the experiments reported here, we studied purified human skeletal muscle CA III for its capacity to regulate cell growth and extracellular matrix formation in a variety of connective tissue cells. Synovial fibroblast culture experiments showed that unlike CA I and CA II, CA III stimulated synthesis of ^{14}C-labeled hyaluronic acid in a dose-dependent manner ($P < 0.001$). This finding is yet another physiological difference between the CA III and the CA I, CA II isoenzymes. A thorough analysis of the structural differences between the active sites of CA I, CA II, and CA III revealed that the residues Lys-64, Arg-67, and Arg-91 are unique to CA III (1) and may be responsible for the differences in its CO_2 hydratase activity and sulfonamide inhibition kinetics. Our data, however, seem to indicate that these residues are not required for its HA stimulatory function since complete tryptic digestion of CA III did not eliminate this activity. The deduced amino acid sequence for human CA III shows 62% homology with human CA II and 54% with human CA I when pairwise amino acid comparisons are made (2). At the level of tryptic peptides the degree of homology decreases to 30–35% and to 0% in 29 peptides from two to seven amino acid residues long. These are potential

TABLE IV. [^{3}H]DNA SYNTHESIS IN SYNOVIAL, DERMAL, AND MUSCLE CELLS

Additive	Muscle cells	E/C[a]	Synovial cells	E/C[a]	Skin cells	E/C[a]
0.15 *M* NaCl	7694 ± 295[b]	—	4331 ± 313	—	11412 ± 858	—
CA III[c]	7489 ± 2165	0.97	6242 ± 156	1.4[d]	10496 ± 4254	0.92
CTAP-III[d]	52881 ± 2887	6.9[d]	61008 ± 6776	14.0[d]	110767 ± 5369	9.7[d]

[a] E/C, experimental/control.
[b] Numbers represent CPM/10^4 cells mean ± SD of four cultures.
[c] 1.2×10^{-5} M.
[d] $P = <0.001$.

TABLE V. QUALITATIVE COMPARISON OF GLYCOSAMINOGLYCAN (GAG) SYNTHESIS IN FIBROBLAST CULTURES[a]

GAG and enzyme	Additive	Synovial[b]	Muscle[c]
[^{14}C]GAG + S-hyaluronidase	0.15 *M* NaCl	84 ± 2.5	81 ± 4
	CA III	84 ± 7	76 ± 3
	CTAP-III	86 ± 5	80 ± 8
^{35}GAG + chrondroitin AC	0.15 *M* NaCl	61 ± 10	63 ± 13
	CA III	71 ± 7	65 ± 7
	CTAP-III	81 ± 6	54 ± 20
^{35}GAG + chondroitin ABC	0.15 *M* NaCl	65 ± 11	62 ± 9
	CA III	72 ± 7	52 ± 13
	CTAP-III	80 ± 5	63 ± 16

[a] Numbers represent mean percentage hydrolysis of total label ± 1 SD.
[b] *n* = three independent synovial cell strains.
[c] *n* = three independent muscle cell strains.

domains for the nonenzymatic functions of CA III. To elucidate this important issue will require the study of sufficient quantities of the tryptic CA III peptides.

Many *in vitro* studies have appeared concerning HA formation in synovial connective tissue cells in different regulatory settings. They have indicated that hvaluronate synthesis can be stimulated by several agents such as CTAPs (11), bacterial endotoxins (13), increased temperature (14), and serum (14). Stimulation of HA synthesis by serum and a few of the CTAPs is known to be associated with an increase of the HA synthetase complex (15, 16); whether a similar mechanism is operative for CA III is unclear. The CA III effects noted on synovial S-GAG metabolism may in fact depend on a different pathway of intracellular processing of these latter macromolecules, perhaps at the level of core protein synthesis.

Evidence has accumulated regarding the characterization and role of the extracellular matrix of muscle in developing chicks (see (17) for review). It has been reported that GAGs synthesized by chick embryo skeletal muscle fibroblasts differ from those formed by multinucleated myotubes (18). The latter exhibit more and shorter heparin sulfate chains and less hyaluronate deposition in their cell-associated GAG pool (18). It appears that the released collagen types also differ in duplicating multinucleated myoblasts (type V) from those secreted by resting chick myotubes (types I, III, V) (19). Studies in developing chick muscle (20) suggest that a fibroblast–myotube interaction is necessary for the formation of a basement membrane sheath. Other authors have suggested the release of soluble factors as a potential mechanism (21). On the other hand, little is known concerning the regulation of connective tissue formation in human muscle fibroblasts. The current finding showing that normal human muscle fibroblasts have the capacity to modestly increase S-GAG formation, but not HA, in response to the muscle-derived CA III agonist where it constitutes approximately 1–2% of the soluble protein (8) is an unusual response that requires further study. This finding coupled with the observation that muscle fibroblasts respond to a major platelet-derived growth factor, CTAP-III, are new data and may provide a useful *in vitro* system to analyze the role of human growth factor proteins in the differentiation of skeletal muscle.

Recent radioimmunoassay measurements of CA III in the plasma of patients with acute and chronic myolytic states (22) have shown that the quantitative detection of the isoenzyme may be a useful parameter for monitoring muscle destruction, perhaps with better specificity than creatine kinase (22). It would be of interest to measure CA III in the plasma of patients with dermatopolymyositis, an idiopathic inflammatory myopathy associated with muscle destruction (23) and polyarthritis (24), in an attempt to monitor response to therapy and perhaps as a prognostic marker. This has particular importance in patients with normal serum creatine kinase levels in spite of obvious clinical disease activity (23).

The increased responsiveness of normal synovial fibroblasts to CA III raises interesting questions about the potential role (if any) of this muscle product in the synovitis frequently seen with inflammatory diseases of muscle.

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