Cloning, Characterization and Tissue Specific Expression of Amur Tiger (Panthera tigris altaica) IGF-I

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Received January 10, 2006; Accepted April 21, 2006; Online Publication, August 23, 2006
[doi:10.1271/bbb.60008]

Insulin-like growth factor I (IGF-I) plays an important role in regulating gonad function, which is essential for normal reproduction in animals, especially in sexual receptivity and reproductive behavior. In this study, a cDNA encoding Amur tiger (Panthera tigris altaica) IGF-I was isolated from liver total RNA using RT-PCR. The IGF-I cDNA of Amur tiger (ATIGF-I) was highly homologous to that of other animals, 84.8% to rat, 93.7% to human and horse. Alignment analysis showed that the cysteine residues and many amino acid residues of putative mature ATIGF-I are highly conserved in mammalian species, confirming the high sequence homology observed in other species. DNA encoding the mature ATIGF-I peptide was ligated with pET-DsbA expression vector and highly expressed in Escherichia coli BL21 with IPTG induction. The recombinant proteins expressed existed mostly in the soluble protein fraction, and were purified with metal affinity resins. Western blotting confirmed that the recombinant proteins reacted with antibodies against IGF-I. The results obtained here should be useful for large-scale production of biological active ATIGF-I protein, as well as for further research on growth, development, and reproduction in the Amur tiger. Tissue specific expression of ATIGF-I mRNA in the Amur tiger was examined by reverse transcription-polymerase chain reaction (RT-PCR). The major ATIGF-I mRNA expression tissue was the liver, while medium signals were found in the uterus, ovary, and pituitary, and minor signals were detected in various tissues including the heart, spleen, pancreas, and kidney. The results indicate that IGF-I might play an important role in the reproductive system and in cub development in the Amur tiger.

Key words: Amur tiger (Panthera tigris altaica); insulin-like growth factor-I (IGF-I); cDNA cloning; fusion expression; tissue expression

The Amur tiger is the largest of the five remaining sub-species of tiger. The wild Amur tiger (Panthera tigris altaica) is found only in northeast China, far east Russia, and North Korea. The dual threats of illegal poaching and habitat destruction have brought this species to the brink of extinction, with a population of about 450 in the world and 20 in China.1,2 Although there has been some success in captive tiger breeding,3 many problems have yet to be solved. First, the tiger usually has only one mate, and forced breeding often does not bring about successful pregnancy. Second, even if the female tiger is in estrus and willing to mate, a similar problem remains if the male tiger is reluctant to mate.4,5 Although breeding difficulties in the male can be solved temporarily by injection or oral administration of male hormones, this is not a suitable option, as it can bring about hormone dependency. Furthermore, the administration of male hormones can cause premature cessation of sexual capability in males in their prime.6 Once the female tiger becomes pregnant, it is necessary to provide her with supplemental nourishment in order to improve fetal development; otherwise the survival rate of the new born is likely to be reduced below the average survival rate of only 50%.7,8 Therefore, there is an urgent need to understand the breeding and reproductive biology of the Amur tiger in greater detail.

IGF-I is a key growth factor in many reproductive events. IGF-I signaling is absolutely required for sexual development and attainment of reproductive competence.9 IGF-I cDNA sequences have been cloned among many species, including humans, tilapia (Oreochromis mossambicus), carp, and others.10–12 The amino acid sequences of IGF-I are highly conserved in many reported mammals.13 In female animals, IGF-I is very important in certain reproductive behaviors, and includes administrating LH release, which accelerates the initiation of puberty, managing follicular development,
oocyte maturation, uterine endometrial proliferation, placental function, and preimplantation embryo and fetal development.\textsuperscript{14–16} IGF-I is also important in mammary gland development during late pregnancy, lactation, and the involution period. IGF-I can also regulate female sexual receptivity and reproductive behavior by cooperating with estradiol receptors.\textsuperscript{17–19} In addition, a higher IGF-I concentration in sperm can increase scrotal circumference, percent motile sperm cells, calving rate, sperm motility, and fertilization in male animals. By acting on Leydig cells, IGF-I directly stimulates gonadotropin release, which induces testosterone formation and potentially influences reproductive behavior.\textsuperscript{20,21}

Preservation of endangered wildlife, including captive breeding and improved fertility and fetal development, are of significance for ultimate realization of field breeding, feral promotion, and expansion of the wild population of Amur tigers.\textsuperscript{22,23} Multiple treatments with exogenous hormones can stimulate sex organ development and activity in endangered species. For example, purified porcine gonadotropin can stimulate ovarian activity in tigers.\textsuperscript{24} Extraneous supplements of recombinant human IGF-I into the serum has improved the developmental capacity of oocytes in prepubertal cattle.\textsuperscript{25} Therefore, many scientists continue to have an interest in functional research on IGF-I in the reproductive system. Cloning and/or expression of several kinds of hormones of endangered animals have been reported, and might offer recombinant hormones to increase reproductive rates, and reproductive system development and lessen infant mortality in captive animals.\textsuperscript{26–28}

In the present study, we cloned and sequenced full-length sequences of the IGF-I gene from the Amur tiger (ATIGF-I). Also we successfully expressed and purified the recombinant protein in prokaryote \textit{E. coli}. In addition, the evolution status of ATIGF-I was analyzed according to the conserved region of IGF-I among different species. Tissue-specific expression of IGF-I mRNA indicated that the expression level was high in the liver, medium in the ovary, pituitary, and uterus, and low in the heart, spleen, kidney, and pancreas. This result implies that IGF-I might be an important component of the animal reproductive system. This study offers a foundation for further research on the reproductive biology of the Amur tiger.

**Materials and Methods**

**Materials and extraction of total RNA.** The tissues (liver, pituitary, kidney, heart, spleen, pancreas, ovary, and uterus) used came from a newborn female Amur tiger which died a natural death at Chengdu Zoo. The tissues were immediately frozen in liquid nitrogen. Total RNAs were extracted from the frozen tissue with Trizol reagent (GibcoBRL, Boston, MA) according to the standard protocol. The total RNAs were dissolved in water treated with DEPC and stored at $-80\,^\circ\text{C}$ until used.

**IGF-I cDNA amplification by RT-PCR.** Corresponding to conserved regions of mammals, using the Primer Premier 5.0 program, primers (ATIGF-A: 5'-TTGCTC-TAT TATT(C) CTGCTA-3'; ATIGF-B: 5'-CTTCTT-ACATT(C)CTGTF AGTTC-3') for IGF-I cloning were designed. A reverse transcription system (Promega, Madison, WI) was used to synthesize the first liver cDNA strand according to the standard protocol with 1 mg of total liver RNA and 15 U AMV reverse transcriptase. Then the Amur tiger liver IGF-I cDNA was amplified by 30 cycles of PCR using 1 \mu l of single-stranded cDNA, 1.5 \text{mM} \text{MgCl}_2, 300 \text{pm} primers, and 2.5 U Taq Plus polymerase (MBI Fermentas, Vilnius, Lithuania). Following 5 min of denaturation at 94\,^\circ\text{C}, PCR was conducted for 30 cycles of 1 min each at 94\,^\circ\text{C}, 40 sec of annealing at 55\,^\circ\text{C}, and 40 s of extension at 72\,^\circ\text{C}, followed by 20 min at 72\,^\circ\text{C}. The PCR product was electrophoresed in 1\% agarose gel. All PCR reactions were performed with the Gene Amp PCR System 9700 (Perkin-Elmer, Boston, MA).

Purified PCR products were ligated into the multiple cloning site of pGEM-T easy vector (Promega, Madison, WI), then transformed into \textit{E. coli} Top 10 F\textsuperscript{ competent cells. Recombinant clones were selected using the blue/white-screening method. Following PCR verification, positive clones were sequenced with universal primers on an ABI 377 automatic sequencer (Perkin-Elmer, Boston, MA) and at least three independent PCRs and sequencings were conducted to avoid possible errors during PCR.

**Homology analysis.** Clustal X 1.81 and Genedoc 3.2 software were employed to perform and display the multiple alignments for IGF-I amino acid sequencing of the Amur tiger with other IGF-I sequences of different mammal species.\textsuperscript{29,30} Blast searches in NCBI (http://www.ncbi.nlm.nih.gov/blast/) were used to show the identity of these IGF-I sequences.

**Construction of expression plasmid.** DNA fragment encoding the mature peptide of ATIGF-I was obtained by PCR amplification with oligonucleotides primers (ATIGF-C: 5'-TTGGATCCGAGACACTCTGTT-3' containing \textit{Bam HI} site; ATIGF-D: 5'-TTGGCTAGCGGACTTTGCAGGT-3' containing \textit{Nhe I} site) designed from the Amur tiger IGF-I sequence we cloned. The amplified fragment was ligated into pGEM easy T-vector (Promega, Madison, WI) and transformed into competent cells of \textit{E. coli} Top 10 F\textsuperscript{ competent cells. Following PCR verification and enzymatic digestion with \textit{Nhe I} and \textit{Bam HI}, the purified digested fragments were subcloned into the multiple \textit{Nhe I} and \textit{Bam HI} sites of pET-DsbA expression vector.\textsuperscript{31} Nucleotide sequencing was carried out to ensure the absence of PCR-induced mutation. The plasmid constructed was dominated by pET-DsbA-ATIGF-I and transformed into competent cells of \textit{E. coli} BL21 (DE3) plysS for fusion expression.
Expression of fusion protein. A single colony of BL21 containing the constructed plasmid was grown overnight at 30°C in LB medium (120 μg/ml ampicillin). Cultures were then diluted in LB medium (120 μg/ml ampicillin) and grown at 37°C, 180 rpm for 2.5 h until OD$_{600}$ reached approximately 0.5. Expression of fusion protein was initiated by the addition of 100 μg/ml isopropyl-β-D-thiogalactoside (IPTG) (Sigma-Aldrich, St. Louis, MO) and then the cultures were grown for 1.5 h at 35°C, 180 rpm. After induction, 1.5 ml culture was centrifuged at 10,000–12,000 g for 15 min at 4°C. The cell pellet was dissolved in 100 μl 1 × SDS sample buffer, heated at 100°C for 5 min, and electrophoresed on 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli with Mini-PROTEAN II Electrophoresis Units (Bio-Rad, Hercules, CA). The same colony without IPTG induction served as a control.

Purification of the fusion proteins. Purification of the soluble recombinant protein was achieved by chromatography on BD TALON™ Metal Affinity Resins (Clontech, Palo Alto, Calif.) following the manual provided. Two hundred ml of cultured cells induced as described above was harvested by centrifugation at 3,000 g for 15 min at 4°C. The cell pellet was re-suspended in 2 ml of chilled 1 × equilibration/wash buffer containing 0.75 mg/ml lysozyme. After incubation at room temperature for 20 min and sonication, the cell extract was centrifuged at 10,000–12,000 g for 20 min at 4°C to pellet any insoluble material. Filtered supernatant was added to the resin. After allowing the polyhistidine-tagged protein to bind the resin, the resin column was washed once with 5 bed volumes of 1 × equilibration/wash buffer containing 10 mM imidazole. The polyhistidine-tagged protein was eluted by adding 5 bed volumes of elution buffer containing 150 mM imidazole to the column. The elution was collected in 500-μl fractions. Spectrophotometric and SDS/PAGE analyses were used to determine which fraction contained the majority of the polyhistidine-tagged protein.

Western blotting. The purified recombinant fusion proteins were electrophoresed on 12% SDS–PAGE. Following electrophoresis, proteins were transferred to Hybond N+ membrane. After the membrane was dyed with ponceau, the protein binding sites of the membrane were blocked in a plate with TBST solution (500 ml TBST, 5% skim milk) for 2 h. After pouring off the TBST solution, primary antibody (polyclonal rabbit anti-human IGF-I, Boster, Wuhan, China) at a dilution of 1:500 was added and the plate was slightly shaken for 2 h, and then the membrane was rinsed with TBST. Then a secondary antibody (goat anti-rabbit IgG alkaline phosphatase) (Boster, Wuhan, China) at a dilution of 1:2,000 was added to the membrane. The plate was slightly shaken for 2 h, and was rinsed with TBST, followed by 5 Bromo 4 Chloro 3 indolylphosphate (BCIP)/Nitroblurtetrazolium (NBT) substrate. The plate was incubated until the signal was clearly visible, and color stopped appearing, and then it was rinsed with tap water. The rhIGF-I (recombinant human IGF-I) purchased (BBI, Toronto, Canada) with a molecular mass of 7.7 kDa served as positive control.

Examining tissue-specific expression of IGF-I. Purity was determined and quantitative analysis of total RNA was done by the A260/A280 and A260/A230 ratio. First-strand cDNA synthesized from the pituitary, heart, spleen, kidney, ovary, uterus, pancreas, and liver was used as template for PCR. The primer pairs used for PCR were 5’GGACCGAGACACTCTGT-3’ and 5’GGCGGACTTGGCCAGGCTT-3’ to amplify a 521 bp fragment of IGF-I cDNA. Primer sequences (5’GC-ATCGTGGAGGGACTTATG3’ and 5’GCCGCTTTCACACCCTTCT3’) were designed from the bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA sequence (GenBank accession no. U85042) as a positive control to amplify a 290 bp product. All the PCR-amplification was performed using the following cycling conditions: 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min for 24 and 26 cycles, followed by 10 min at 72°C in a 100 ul reaction volume (RT-PCR Kit, MBI Fermentas, Vilnius, Lithuania). PCR products (10 μl) were separated by agarose gel electrophoresis, and one PCR product was selected for further sequencing to verify the GAPDH gene.

Results

Characterization of the Amur tiger IGF-I gene Total RNA was successfully extracted from Amur tiger liver tissue and reverse transcribed to cDNA, and then the ATIGF-I cDNA was amplified by RT-PCR. PCR products of 521 bp were obtained. After purification and subcloning into PGEM-T, the positive clones were sequenced. Blast searches in NCBI confirmed that the sequences obtained represented mammalian coding region sequences of IGF-I. This liver ATIGF-I nucleotide sequence has been submitted to Genbank under accession no. AY635911. The ATIGF-I cDNA contains an open reading frame of 153 aa (Fig. 1), including a leader signal peptide of 48 aa (1 ± 48), the mature protein of 70 aa (49 ± 118) in the B, C, A and D domains, and an extended carboxy-terminal polypeptide of 35 aa (119 ± 153) in the E domain. The predicted molecular mass of the mature protein was 7.7 kDa using ANTHEPROT software package ver 4.5. Six conserved cysteines were found in Amur tiger IGF-I peptide that formed three disulfide bonds: Cys$^{54}$–Cys$^{96}$, Cys$^{86}$–Cys$^{109}$, and Cys$^{85}$–Cys$^{100}$.

Homology analysis and blast searches Blast searches revealed that ATIGF-I shares a high level of similarity to IGF-I and proinsulin sequences from other species, including human, horse, rat, rabbit, cow, ostrich, turkey, zebrafish, and perch. The similarity
Characterization of Amur Tiger IGF-I

The predicted amino acid sequence of the IGF-I among species indicated that the primary structure of IGF-I has been extensively conserved throughout vertebrate evolution. Moreover, the conserved structure shared common domains in mature peptide in mammalian species. But, IGF-I is one of the molecules with a variable evolutionary rate, and thus there is low similarity in signal peptides and the E domain among species. This indicates IGF-I has a long evolutionary history. For example, the mature peptide of ATIGF-I is completely identical to the human, which has led to suggestions that these proteins originated from a common precursor. A rooted phylogenetic tree was constructed using maximum parsimony methods by comparison of the mature ATIGF-I amino acid sequence with other species, which suggests that mature ATIGF-I evolved from a common ancestral origin (rat) and diverged into the various species (Fig. 3). From this phylogenetic tree, 10 species are clustered into three distinct groups. The first group includes the zebrafish and the perch. The second group includes the ostrich and the turkey. The third group is a mammalian group including the rat, rabbit, cow, horse, human, and tiger. Within the IGF-I molecular tree, the tiger’s is closest to the human’s.

Expression and purification of the fusion proteins
DNA encoding the Amur tiger IGF-I mature peptide

Fig. 1. Nucleotide and Putative Amino Acid Sequences of Amur Tiger IGF-I.

The single underlines indicate the primer sequences and the double underlines indicate the sequences of signal peptides. The broken underlines identify the sequences of mature peptides. The conserved cysteines (Cys) are boxed by open rectangles and the stop codon is indicated by the asterisk. The predicted domains of coding regions are signal peptide (amino acid number 1–48), B domain (49–77), C domain (78–90), A domain (91–110), D domain (111–118), and E domain (119–153).

is 93.7%, 93.9%, 84.8%, 89.6%, 92.4%, 82.5%, 81%, 64.9%, and 68.9% respectively at the nucleotide sequence level; this corresponds to 97.4%, 97.6%, 89.8%, 91.2%, 95.4%, 85%, 83.7%, 63.5%, and 66.4% respectively at the amino acid level. The amino acid sequences aligned with other species are provided in Fig. 2. The mammalian IGF-I mature peptides contain a total of 70 amino acid residues. The mature peptide in Amur tiger was completely identical to those of human and horse. The Amur tiger and the rat had different amino acid residues in the mature peptide at positions 69, 84, and 116. Alignment analysis also indicated that all cysteine residues of the deduced mature peptide are completely conserved in the listed mammals’ IGF-I sequences, but, there was a low similarity in the signal peptide domain and E domain among the mammals.

The evolution of ATIGF-I among species

The predicted amino acid sequence of the IGF-I among species indicated that the primary structure of IGF-I has been extensively conserved throughout vertebrate evolution. Moreover, the conserved structure shared common domains in mature peptide in mammalian species. But, IGF-I is one of the molecules with a variable evolutionary rate, and thus there is low similarity in signal peptides and the E domain among species. This indicates IGF-I has a long evolutionary history. For example, the mature peptide of ATIGF-I is completely identical to the human, which has led to suggestions that these proteins originated from a common precursor. A rooted phylogenetic tree was constructed using maximum parsimony methods by comparison of the mature ATIGF-I amino acid sequence with other species, which suggests that mature ATIGF-I evolved from a common ancestral origin (rat) and diverged into the various species (Fig. 3). From this phylogenetic tree, 10 species are clustered into three distinct groups. The first group includes the zebrafish and the perch. The second group includes the ostrich and the turkey. The third group is a mammalian group including the rat, rabbit, cow, horse, human, and tiger. Within the IGF-I molecular tree, the tiger’s is closest to the human’s.

Expression and purification of the fusion proteins
DNA encoding the Amur tiger IGF-I mature peptide
was ligated with pET-DsbA expression vector and used to transform *E. coli* BL21 (DE3) plysS. Expression of the IGF-I encoded in the vector was induced with IPTG. Cells and elution fractions were analyzed on SDS–PAGE gel (Fig. 4). It was found that a fusion protein band with a molecular weight of 33 kDa was obtained quite definitely, which presumably was comprised of an ATG-6His DsbA and an Amur tiger mature IGF-I peptide, but was not as strongly observed in the samples without IPTG induction. Solubility analysis suggested that the expressed DsbA-ATIGF-I existed mostly in the soluble protein fraction. Purification fractions eluted by 150 mM imidazole showed a unique fusion protein band.

**Fig. 2.** Multiple Alignments of IGF-I Putative Amino Acids Sequences from Different Vertebrates.

The sequences have been shaded to emphasize the identities (black) and similarities (from dark gray to shallow gray). Conserved residues are shown in the last line of each block (the absolute conserved residues are capitalized and the highly conserved residue are in lower case). The sequences obtained are: Amur tiger (Genbank no. AY635911), human (AB209184), horse (U85272), rat (D00698), cow (X15726), rabbit (U75390), ostrich (AB035804), turkey (AF074980), zebrafish (NM_131825), perch (AJ586908).

**Fig. 3.** Rooted Phylogenetic Tree Based on Amino Acid Sequence of Mature IGF-I Constructed by the Maximum Parsimony Method. Numbers indicate bootstrap values.

Western blotting

The results of western blotting (Fig. 5) revealed that the observed molecular mass of the recombinant proteins was in accordance with the estimated molecular weight (33 kDa) of the DsbA-ATIGF-I fusions, which indicates that the purified recombinant fusion proteins...
from the induced cell cultures had IGF-I immunoreactive antigen activity.

**Tissue-specific expression of IGF-I gene transcript in Amur tiger**

Tissue specific expression of IGF-I gene transcripts in various organs of the Amur tiger was analyzed by RT-PCR. Sequencing of the 290 and 521 bp PCR products confirmed bovine GAPDH and ATIGF-I respectively. Expression of IGF-I mRNA in various Amur tiger tissues, as determined by RT-PCR, is shown in Fig. 6. Evidence of IGF-I expression is indicated by the presence of a 521 bp product. IGF-I mRNA expression levels were high in the liver, medium in the ovary, pituitary, and uterus, and low in the heart, spleen, kidney, and pancreas. As a positive control of RNA isolation and cDNA synthesis from each tissue, GAPDH (290 bp) was amplified from each tissue tested using bovine GAPDH-specific primers. The GAPDH mRNA levels remained unaffected and showed constant expression in the samples studied both at 24 PCR cycles and at 26 PCR cycles (Figures not shown).

**Discussion**

In this study we determined the characterization of Amur tiger IGF-I and expressed the IGF-I protein in E. coli. Sequence comparisons showed that the structures of Amur tiger IGF-I are very similar to those of other mammals. The 70-aa IGF-I we characterized in the Amur tiger contained all the features of IGF-I peptides with B, C, A, and D domains and conservation of the six cysteine residues involved in the tertiary structure, but not in the E-domain or the signal peptide, suggesting that some residues in the conserved regions might influence the structure and function of the prohormone. For example, Glu51, Thr52, Gln63, and Phe64 are involved in IGF binding with IGFBP.35) In the A-domain, Arg104, Tyr108, Phe97, and Ser99 are also involved in IGF binding with IGFBP.36) Moreover, the sequences of the B domain in IGF-I play an important role in guiding its unique bifurcating folding behavior.37,38) Mature Amur tiger IGF-I was 100% identical to that of human and horse and was highly conserved in those of the other species. The B- and A-domains of Amur tiger IGF-I showed a high degree of sequence identity. Furthermore, IGF-I contained carboxyl-terminal sequences called D- and E-domains.39) The E-domain is removed as a post-translational modification to produce mature IGF.40) The results showed that IGF-I was conserved over a long period of evolutionary history, while there was a high evolution rate in domain E and the signal peptide.

Sequence alignments indicated that IGF-I evolved very slowly by comparison of the ubiquitous IGF-I mature peptides obtained from different species (Fig. 2). The rooted phylogenetic tree is based on the predicted IGF-I mature peptide sequences analyzed using the UPGMA clustering method (Fig. 3). Substitutions and deletions occurred in the mature peptide IGF-I peptide during evolution. In comparison with the mature peptides among mammalian species, the B and A domains of IGF-I have been highly conserved (more than 90% identity), but the signal peptide, C, D, and E domains of IGF-I have been variable among mammals and other chordates (67%–100%). This study suggests that IGF-I might have an important role in the evolutionary rate of mammalian species.

**E. coli** is still a preferred expression host choice for eukaryotic genes because of its convenience, economy, rapid growth, and high-yield advantages, but most proteins aggregate into insoluble and inactive inclusion bodies when the yield is high in bacterial cells. In the present study, the Amur tiger IGF-I mature peptide proved to be a single peptide without glycosylation with three disulfide bonds, and it was successfully expressed
in the *E. coli* system. DsbA has been widely used in constructed plasmid to catalyze the formation of the disulfide bonds in *E. coli* in order correctly to reform the disulfide bonds and obtain the active renatured proteins.\(^41,42\) The modified DsbA mutant was used successfully to produce high levels of soluble and active fusion proteins, such as humanIGFBP-3 and mEGFBP.\(^43\) In the present study, pET-DsbA is an inducible fusion expression vector designed to increase production combined with a 5′ end of a 6His Tag, an efficient T7 promoter, a thrombin cleavage site to separate the DsbA\(^{mut}\), and the His tag from the targeted expressed protein. In addition, 35 °C was selected as the optimum inducing temperature to get the highest yield of soluble proteins of interest, despite some recombinant proteins in inclusion bodies (Fig. 4). Western blotting further confirmed that the purified soluble recombinant proteins from the induced cell cultures bore IGF-I immuno-reactivity (Fig. 5). The eluted purified proteins showed a unique band with an estimated molecular weight of 33 kDa. The high-yield expression system and the purification techniques of the soluble recombinant IGF-I proteins gave us the opportunity to study further the biological function of recombinant Amur tiger IGF-I in *vivo*.

Tissue-specific expression of IGF-I gene mRNA was detected in a naturally deceased newborn female Amur tiger whose reproductive organs were still developing. The major site of IGF-I expression was in the liver, while medium levels of mRNA were detected in the pituitary, ovary, and uterus. Our results indicate that IGF-I might play an important role in animal sex organ development. More information is required for a complete understanding of the complicated local functions of IGF-I in tissues. In the PCR detection procedure, moderate signals were observed in the immature ovary and uterus tissues at 26 PCR cycles as compared with amplification in other tissues, and the signal appeared more clearly at 28 PCR cycles (Data not shown). We used the bovine GAPDH gene as the internal control in this study. To act as a good internal control, a gene must have a constant basal level of expression that is consistent, non-regulated, and independent of the cell cycle. GAPDH expression has been found by a number of studies to be influenced by certain factors such as growth hormone and alcohol.\(^44\) But GAPDH expression was not different in the eight tissues studied, and this indicates that GAPDH was a suitable internal control to reflect tissue expression in this study.

Since the Amur tiger is an endangered species, several university laboratories and local research stations are conducting research on natural populations and in addition are studying techniques for endangered-animal breeding.\(^45,46\) The sequence of the Amur tiger IGF-I cDNA obtained in the present study confirms high sequence identity among species, suggesting that these maintained growth factors played an important role during evolution. The characterization of IGF-I is important in understanding the mechanisms of reproductive biology, including sexual receptivity and the reproductive behavior of this species, and might perhaps contribute to the amplification of the wild Amur tiger population. Moreover, we have developed an expression system in *E. coli* to overproduce soluble recombinant Amur tiger IGF-I. The purification and production of recombinant ATIGF-I might be useful for study of its biological activity and might offer an assisted way to improve milk production, growth rate, reproduction, and breeding in the Amur tiger.

Acknowledgments

This research was supported by the Chengdu Research Fund for Giant Panda Breeding (Grant no. 2000-19), the National Basic Research Project of China, the Eastbay Zoological Society of Oakland, California, and the Oakland-China Wildlife Preservation Foundation. We thank everyone at the Reproductive and Genetic Laboratories at Chengdu Giant Panda Breeding for useful suggestions and technical assistance.
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