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Calcitonin gene expression in normal human liver

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Immunoreactive calcitonin (CT) is present in liver. This could represent hormone synthesized by liver cells, degraded or bound to specific receptors reported in this organ. We report here that the calcitonin gene is expressed in liver We proved this by demonstrating, by PCR amplification using specific primers, the presence of calcitonin messenger in human liver and in primary cultures of human hepatocytes and detected by radioimmunoassay CT in hepatic tissues and cells. The synthesis of hormone by liver that also possesses specific receptors for CT favors the presence of an autocrine or paracrine system involving calcitonin in this organ.

Autocrine; Paracrine; Biosynthesis; Calcitonin mRNA (human liver); PCR

1. INTRODUCTION

The primary source of endogenous calcitonin (CT) secretion is the parafollicular C cells which derive from the embryonal neural crest and migrate to the thyroid gland in mammals and in the ultimobranchial body in non-mammalian species [1]. Calcitonin exerts a hypocalcemic and hypophosphatemic effect, inhibits bone resorption, and stimulates renal clearances of phosphate, calcium, and sodium. The hormone acts through specific receptors present in bone and kidney. In these tissues CT receptor is coupled with the activation of adenylate cyclase and formation of cyclic AMP.

Binding sites of the hormone have also been discovered in many tissues, such as the central nervous system, the pituitary gland [2], lymph cells tumoral cell lines and liver [3]. In this organ CT increases calcium content [4] by mobilization of intracellular calcium. The presence of immunoreactive calcitonin is reported in many tissues [5] and particularly in liver [6]. Thus the molecule detected in liver could represent hormone bound to its specific receptors or degraded by the organ. It also could be the result of active synthesis by one or several of the cell types present in liver. We have therefore measured CT levels in human liver and in primary cultures of human hepatocytes. We also searched for CT-specific messenger by polymerase chain reaction.

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Abbreviations: CT, calcitonin; hCT, human calcitonin; Rnase, ribonuclease A; PCR, polymerase chain reaction, BSA, bovine serum albumin; SSC, sodium saline citrate.

2. MATERIALS AND METHODS

2.1. *Tissue*

Normal tissue obtained from liver resected before hepatic transplantation was immediately frozen in liquid nitrogen and stored at −80°C until extracted.

2.2. *Cell isolation and culture*

Human liver samples were obtained from patients undergoing partial hepatectomy All procedures were performed in compliance with French laws and regulations and were approved by the National Ethics Committee Hepatocytes were isolated by the two-step collagenase perfusion method [7]. Subsequently, cells were seeded at a density of 10^7 hepatocytes/80 cm^2 flask in 10 ml of a mixture containing 75% minimum essential medium and 25% medium 99 supplemented with 10 μg/ml bovine insulin, 0 2% bovine serum albumin and 10% fetal calf serum The medium was renewed daily and supplemented with 7×10^{-5} hydrocortisone hemisuccinate.

TT cells a medullary carcinoma cell line producing high levels of CT were cultured in RPMI, 10 mM HEPES, 6 mM glutamine, supplemented with 10% foetal calf serum. The medium was changed every two days and the cells were harvested at confluence.

2.3. *RNA extraction*

Total RNA was extracted from liver and TT cells by a guanidine thiocyanate chloride method [8]. Total RNA was prepared from hepatocytes either immediately or after 4 days culture by Chirgwin's or Chomzinsky's methods [9,8]. All extracts were immediately frozen at −80°C or stored at −20°C in 75% ethanol until used.

2.4. *Polymerase chain reaction*

Polymerase chain reaction cDNA was synthesized from 2 μg of total RNA. The reaction mixture had a final volume of 20 μl, and contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, nuclease free BSA (1 mg/ml), 20 U of RNAsin, 200 U of reverse transcriptase (BRL), 1 mM of each dNTP and 50 pmol of a 3′ oligo dT primer. Annealing was performed during 10 min at 23°C and primer extension during one hour at 37°C. The reaction was then diluted to 100 μl with the same buffer containing 50 pmol of each specific primer and 2.5 units of *Taq* polymerase (Eurobio). Amplification was performed during 25 cycles. 30 s at 95°C (denaturation), 30 s at 55° C (annealing)

and 30 s at 72°C (extension). Control reactions were performed as follows: Positive control with total RNA extracted from TT cells and negative ones with the liver, hepatocytes or TT cells total RNA in the absence of reverse transcriptase or after digestion with RNAase A (1 mg/ml) 1 h at 37°C

2 5. *Analysis of PCR products*

Amplified products were analyzed by electrophoresis in 1.2% agarose gel, visualized by ethidium bromide, transfered to Nylon membrane (NEN).The amplified cDNA was hybridized with a CT-specific probe (exon 4: CT coding sequence) radiolabelled with ^{32}P using a random priming method (Specific activities of 10^8 cpm/μg DNA). The membranes hybridized at 42°C in presence of 50% formamide were washed at 55°C, twice in 2 × SSC for 15 min, 2 × SSC containing 0.1% SDS for 30 min, and 0.1 × SSC for 15 min. Autoradiography was performed at −80°C with intensifying screens overnight.

2.6 *Sequencing of PCR products*

After electrophoresis, the specific band was cut from the gel lane corresponding to liver tissue. The gel slice was frozen in a Spin-X tube (Costar) at −20°C during 20 min. DNA was collected by centrifugation at 12,000 × *g* during 15 min, dissolved and ethanol precipitated then resuspended in H_2O.

The amplified products were submitted to PCR with only one internal antisense primer (p45 or p91) labeled with [γ-^{32}P]ATP and Taq DNA polymerase, to generate single stranded template for DNA sequence analysis by the dideoxynucleotide chain termination method (ds DNA cycle Sequencing System BRL): 20 cycles (95°C 30 s, 55°C 30 s, 70°C 60 s) and 10 cycles (95°C 30 s, 70°C 60 s)

2.7. *Calcitonin content*

2.7.1. Tissue extraction

Liver tissue was extracted with 0.1 M acetic acid, lyophilised and diluted in radioimmunoassay buffer (0.1 M phosphate buffer containing 0.2% human albumin heat denatured and 0.1% sodium azide).

2.7.2. Primary culture extraction

In order to detect both messenger and peptide on the same sample, an aliquot of Chomzinsky's solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) was removed prior to phenol extraction and frozen until assayed.

2 7.3. Radioimmunoassay (RIA)

Radiolabelled human CT (hCT) was obtained by a modification of the Hunter and Greenwood method [10] Assays using antibodies specific for hCT were performed as previously described [11]. In the case of primary cultures non-specific effects of the extraction solution D were controlled by adding equivalent amounts of this solution (2.5 μl) to the standard curve.

3. RESULTS

3.1. *Calcitonin tissue levels*

Levels of CT as estimated by specific radioimmunoassay were 7.5 ng/g wet weight in normal human liver and 0.328 ng/10^6 cells in primary cultures of human hepatocytes. In the RIA, displacement of ^{125}I-hCT by liver or cells extracts was identical to that with synthetic hCT when the specific antibody was used. The addition of 2.5 μl of solution D to the standard curve did not modify the binding of the labelled tracer or its displacement (data not shown).

3.2. *Analysis of PCR results*

The primers used for amplification or sequencing of the PCR products are reported in Table I and in Fig. 1.

Gel electrophoresis of reverse transcription using an oligo (dT)18 primer followed by PCR amplification using specific hCT primers revealed the presence in normal human liver, primary cultures of human hepatocytes and TT cells of a single band stained by ethidium bromide (Fig. 2, panel A). This band had the correct size 291 bases and gave a strong signal after transfer and hybridization (with a CT specific probe, Fig. 2, panel B). No amplification was observed if the aliquots studied were treated with RNase before reverse transcription and amplification.

3.3. *Sequence results*

Direct sequencing of PCR products confirmed that the nucleotide sequence of amplified cDNA was identical to CT mRNA [12] in the region delimited by the primers used (beginning of exon 2 to the end of exon 4) (Fig. 3).

4. DISCUSSION

Our results confirm that human liver contains small amounts of immunoreactive calcitonin molecules. This confirms and extends the previous work of Becker et al.

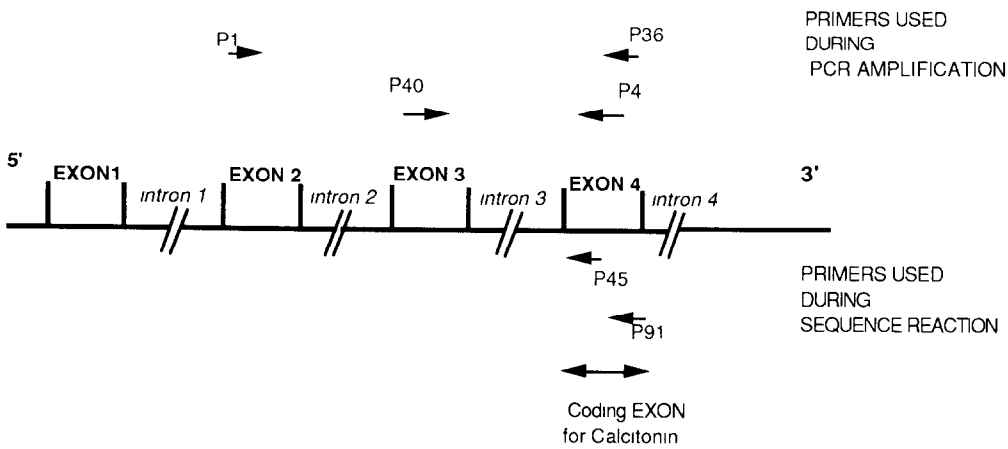

Fig. 1. Position of primers used for amplifying and sequencing on CALC I gene

Table I

Sequence and localisation of the primers used

Primers	Sequence 5′–3′	
P1	CC CCC TTC CTG GCT CTC AGC	Exon 2
P3	GCC CCA GAT CTA AGC GGT GCG G	Exon 4
P4	CCA GGT GCT CCA ACC CC	Exon 4 antisense
P36	CAG CAC AGA GGA GCT CTG AT	Exon 4 antisense
P40	GGC AGC CTC CAT GCA GCA CC	Exon 3
P45	AA CGT GTG AAA CTT GTT	Exon 4 antisense
P91	TT CTC TTC CAA CCT GTG AGT CC	Exon 4 antisense

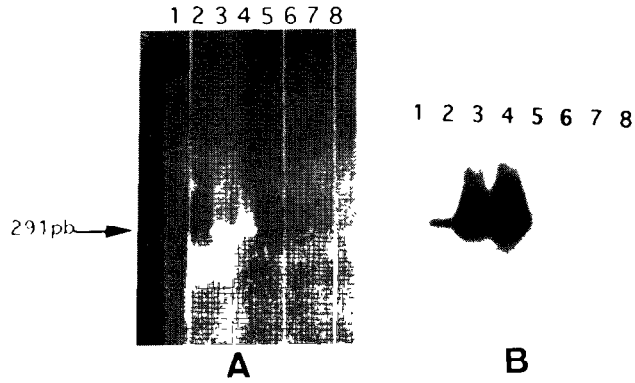

Fig. 2. Panel A: agarose gel analysis of PCR products. Total RNAs extracted from normal human liver, primary cultures of normal human hepatocytes (after 4 days culture) and TT cells, were reverse transcribed and amplified with specific CT primers. One fourth of the reaction was electrophoresed. The 1.2% agarose gel contained 0.5 mg/ml ethidium bromide. Lanes 1,5: negative control without RNA. Lanes 2,6: primary cultures of normal human hepatocyte. Lanes 3,7: normal human liver. Lanes 4,8: positive control (TT cells). Lanes (5,6,7,8): control products resulting from digestion of total RNA by RNase (1 mg/ml) 1 h at 37°C, then reverse transcribed and amplified with specific CT primers P40 and P4 Each sample represents one fourth of the PCR reaction. Panel B: autoradiogram of the Southern blot of PCR products (Fig. 2, panel A) hybridized,with ^{32}P-radiolabeled CT-specific probe.

[6], which showed that the molecule was present in monkey liver at a concentration of 12.30 ng CT/g wet weight. In the human liver we find similar levels 7.5 ng per gram wet weight. After thyroidectomy the level of the hormone, in monkeys, decreased 25-fold to 0.48 ng CT/g wet weight. Thus a large amount of the CT we detected in hepatic tissues could be of thyroid origin and represent hormone bound to its specific receptor or hormonal fragments degraded by the hepatic tissue. However in primary cultures of hepatic cells we found 32.8 ng/10^8 cells roughly the equivalent of 1 gram tissue. This higher level of CT in vitro as compared to in vivo could be due to a higher expression of the CT gene in the in vitro conditions we used. In particular glucocorticoids, which are necessary to maintain a normal metabolic activity in cultured hepatocytes, increase the secretion and levels of CT in some cell lines such as the TT cell line [13]. The capacity of hepatocytes to synthesize calcitonin in vitro suggested that the hormone present in liver in vivo was produced in situ. Direct proof of this was obtained by our demonstration that CT messenger is present both in liver and in primary cultures of hepatocytes.

The specificity of the amplified PCR products was directly established by hybridization with a CT-specific probe and by sequencing the amplified cDNA between the beginning of the exon 2 to the end of the exon 4. The messenger expressed in the liver is polyadenylated as we used oligo dT and reverse transcriptase to obtain the first cDNA copy.

The liver contains in addition to hepatocytes, the major type of cell present, small numbers of other cells such as Kuppfer and ITO cells, fibroblasts, and endothelial cells. The probable source of the hormone is

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   Leu Leu Gln Ala Gly Ser Leu His Ala Ala Pro Phe Arg Ser      14
 1 CTG TTG CAG GCA GGC AGC CTC CAT GCA GCA CCA TTC AGG TCT
   Ala Leu Glu Ser Ser Pro Ala Asp Pro Ala Thr Leu Ser Glu      28
44 GCC CTG GAG AGC AGC CCA GCA GAC CCG GCC ACG CTC AGT GAG
   Asp Glu Ala Arg Leu Leu Leu Ala Ala Leu Val Gln Asp Tyr      42
86 GAC GAA GCG CGC CTC CTG CTG GCT GCA CTG GTG CAG GAC TAT
   Val Gln Met Lys Ala Ser Glu Leu Glu Gln Glu Gln Glu Arg      56
128 GTG CAG ATG AAG GCC AGT GAG CTG GAG CAG GAG CAA GAG AGA
   Glu Gly Ser Ser Leu Asp Ser Pro Arg Ser Lys Arg Cys Gly      70
170 GAG GGC TCC AGC CTG GAC AGC CCC AGA TCT AAG CGG TGC GGT
   Asn Leu Ser Thr Cys Ile Leu Gly Thr Tyr Thr Gln Asp Phe      84
212 AAT CTG AGT ACT TGC ATC CTG GGC ACA TAC ACG CAG GAC TTC
   Asn Lys Phe His Thr Phe Pro Gln Thr Ala Ile Gly Val Gly      98
254 AAC AAG TTT CAC ACG TTC CCC CAA ACT GCA ATT GGG GTT GGA
   Ala Pro Gly Lys Lys Arg Asp Met Ser Ser Asp Leu Glu Arg     112
296 GCA CCT GGA AAG AAA AGG GAT ATG TCC AGC GAC TTG GAG AGA
   Asp His Arg Pro His Val Ser Met Pro Gln Asn Ala Asn Stop    125
338 GAC CAT CGC CCT CAT GTT AGC ATG CCC CAG AAT GCC AAC TAA
380 ACTCCTCCCTTTCCTTCCTAATTTCCCTTCTTGCATCCTTCCTATAACTTGATGC
435 ATGTGGTTTGGTTCCT
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Fig. 3. Partial nucleotide and peptide sequence of liver cDNA to calcitonin mRNA amplified by PCR. The N-terminal, calcitonin and C-terminal peptides are represented in italic.

probably the hepatocyte. We confirmed this directly by our detection of specific CT messenger and peptide in primary cultures of these cells.

The presence of calcitonin and its messenger in liver and the concomitant existence of specific receptors of the hormone favors the presence of an autocrine and or paracrine system of regulation implicating this molecule in the liver. This system could be involved in liver metabolism as the hormone stimulates glycogenolysis, gluconeogenesis [14], fatty acid synthesis and ATP synthesis and lipid metabolism [15].These actions of CT on liver metabolism are mediated through cellular calcium [4,16] and involve Gs proteins [17].

In conclusion we demonstrated the production, by normal human liver of CT. The peptide is expressed in situ by the CALC I gene and could be implicated in an autocrine or paracrine regulatory role involving the metabolic activity of this organ.

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