Local Estrogen Formation by Nontumoral, Cirrhotic, and Malignant Human Liver Tissues and Cells

Luigi A. M. Castagnetta, Biagio Agostara, Giuseppe Montalto, et al.

Cancer Res 2003;63:5041-5045.

[CANCER RESEARCH 63, 5041–5045, August 15, 2003]

Local Estrogen Formation by *Nontumoral,* Cirrhotic, and Malignant Human Liver Tissues and Cells

Luigi A. M. Castagnetta, Biagio Agostara, Giuseppe Montalto, Lucia Polito, Ildegarda Campisi, Annalisa Saetta, Toru Itoh, Bin Yu, Shiuan Chen, and Giuseppe Carruba[1]

Department of Experimental Oncology and Clinical Application [L. A. M. C., A. S., G. C.] and Institute of Internal Medicine [G. M.], University Medical School, Palermo, Italy, Experimental Oncology Unit [L. A. M. C., L. P., I. C.] and Division of Clinical Oncology [B. A.], Department of Clinical Oncology, ARNAS-Civico, 90127 Palermo, Italy, and Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California [T. I., B. Y., S. C.]

ABSTRACT

We have investigated the activity and expression of aromatase enzyme in nontumoral, cirrhotic, and malignant human liver tissues and cells using both chromatographic and reverse transcription (RT)-PCR analyses. After 24- and 72-h incubation of tissue minces or hepatic cell lines with either testosterone or androstenedione as androgen precursor, human hepatocellular carcinoma (HCC) tissues and HepG2 hepatoma cells showed elevated aromatase activity, with estrogen formation rates being 20 and >95%, respectively, as opposed to nontumoral hepatic tissues and nonmalignant Chang liver (CL) cells, where no aromatase activity could be detected. Cirrhotic samples exhibited intermediate enzyme activity. Notably, exposure of HepG2 cells to the aromatase inhibitor Letrozole resulted in a striking decrease of estrogen formation, which became virtually absent at a Letrozole dose of 0.4 nM. RT-PCR analysis revealed markedly lower aromatase mRNA in both CL cells and nontumoral liver tissues, as compared with HepG2 cells and HCC samples. Cirrhotic specimens displayed variable transcript levels, in turn comparable with those observed in nontumoral or HCC tissues. Exon-specific RT-PCR showed prominent expression of exon I.3A-containing message and exon I.4-containing message in CL and HepG2 cells, as in nontumoral and HCC tissues, respectively. The present evidence implies that locally elevated estrogen formation in malignant human liver tissues and cells may have a role in the development and/or maintenance of human HCC, eventually leading to develop alternative strategies for treatment of HCC patients using antiaromatase agents.

INTRODUCTION

Incidence and mortality rates of human HCC[2] have been continuously increasing in recent years to such a point that today, HCC represents the sixth leading cancer and the third most common cause of death from cancer worldwide (1). In our country, mortality rates have been drastically increasing from 4.8 deaths/100,000 in 1969 up to 11/100,000 in 1994, with even greater figures in the South of Italy (Italian ISTAT Istituto Nazionale di Statistica database). Geographical distribution of HCC varies greatly worldwide; however, independent of race and geography, rates in men are at least two to three times those in women, with a more pronounced sexual dimorphism in high-risk regions, including our own. This hints at a potential role for sex steroids in HCC development and progression. On the basis of previous evidence for ERs in primary HCC (2), different clinical trials have investigated the potential impact of antiestrogen (tamoxifen) treatment on the survival of patients having HCC. From these studies, there is evidence that tamoxifen is of no benefit in prolonging survival of HCC patients; this, however, could be partly attributable to the presence of variant ER forms, as described previously (3), or to alternative, nonreceptorial mechanisms potentially involved in growth regulation of both normal and cancer human liver cells by estrogen (4).

The human liver represents a major site for biotransformation, conjugation, and catabolism of gonadal steroids, being featured by the presence of key steroid enzymes, including aromatase. Although the relationship between the aromatase enzyme and development and growth of different human solid tumors has been repeatedly emphasized, only sparse information exists on aromatase in human liver malignancies. In the present work, we have investigated the activity and expression of the aromatase enzyme in both nontumoral and malignant human liver tissues and cells to get insights into its potential role in human HCC.

MATERIALS AND METHODS

Cell Lines. The CL and HepG2 human liver cells were purchased from the American Type Culture Collection (Rockville, MD). Cells were routinely grown in RPMI 1640, supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B), at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were periodically tested for *Mycoplasma* contamination. Cells having a narrow range of passage number (CL, 259–263; HepG2, 78–83) were used for all experiments.

Tissue and Primary Cultures. Tissues from HCC and adjacent, either normal or cirrhotic, liver were obtained from patients undergoing surgical resection. Histologically normal liver tissues were obtained from patients during surgery for colelithiasis. Written informed consent was obtained in all cases. Fresh samples were collected at the surgical theater and immediately transported to cell culture lab in RPMI routine medium on ice. Tissues were scraped to remove most of the soft adipose tissue and carefully minced in small fragments using a scalpel onto a glass Petri dish. Tissue minces were then either resuspended in fresh medium and seeded onto 30-mm Petri dishes to assess steroid metabolism (see below) or subjected to overnight collagenase digestion (2.5 mg/ml collagenase in RPMI medium, w/v) at 37°C in a water bath with gentle agitation. The digested tissue minces were then centrifuged at 1000 rpm for 5–10 min, and the resulting pellets were washed twice, resuspended in fresh RPMI medium, and finally seeded onto Petri dishes for 2–4 h to allow stromal cells to attach to plastic. The floating epithelial cell aggregates were resuspended in medium and transferred to separate Petri dishes, whereas the stromal cell cultures were maintained in fresh medium. Epithelial and stromal cell cultures were left growing undisturbed with a medium change every 3 days. Seventy to 80% confluent cell cultures were then used to assess steroid metabolism under exactly the same experimental conditions used for either cell lines or tissue minces (see below). For RT-PCR analysis of aromatase expression, 18 tissue samples of normal or cirrhotic liver and primary HCC were collected immediately after surgery, snap frozen in liquid nitrogen, and stored at −80°C until analysis.

Androgen Metabolism. The methods and procedures used to assess the metabolic pathways of gonadal steroids by intact cell analysis have been established previously and optimized in our laboratories (5–7). Briefly, long-term cell lines (5×10^5 cells/dish), or primary epithelial and stromal cell cultures, were grown in routine medium. After 24–48 h, cells or tissue cultures were washed twice with PBS-A and incubated in FCS-free, phenol red-free RPMI medium containing 1 nM labeled T ([1,2,6,7-^{3}H(N)]-T, specific activity

Received 3/27/03; revised 5/16/03; accepted 6/3/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Experimental Oncology, Department of Clinical Oncology, ARNAS-Civico, Via Parlavecchio 143, 90127 Palermo, Italy. E-mail: lucashbl@unipa.it.

[2] The abbreviations used are: HCC, hepatocellular carcinoma; ER, estrogen receptor; v/v, volume for volume; DHT, dihydrotestosterone; HPLC, high-performance liquid chromatography; CL, Chang liver; Ad, androstenedione; T, testosterone; LZ, Letrozole; RT-PCR, reverse transcription-PCR.

92.4 Ci/mmol; DuPont de Nemours Italiana SpA, Milan Italy) or Ad ([1,2,6,7-^{3}H(N)]-Ad, specific activity 84.5 Ci/mmol; DuPont de Nemours) as precursor, in the absence or presence of the aromatase inhibitor LZ at 100 nM. Separate experiments were also carried out using increasing T concentrations (from 0.1 to 100 nM). After either 24- or 72-h incubation, medium was transferred to plastic tubes and stored at −80°C until analysis; cells were washed three times using PBS-A and solubilized in 3 ml of 0.1% SDS at 37°C for 15–30 min. Aliquots (100 μl) of the cell lysates were used to estimate DNA content, as described elsewhere (8). Tissue minces were resuspended directly in the above incubation medium containing 1 nM labeled T as precursor and seeded onto 30-mm Petri dishes. After 24-h incubation, minced tissues were resuspended in the incubation medium, transferred to a plastic tube, and centrifuged at 2000 rpm for 5 min. The resulting pellet was gently homogenized using a glass–glass Dounce homogenizer (Kontes Co., Vineland, NJ) in 3 ml of PBS, whereas the supernatant (incubation medium) was transferred to a separate tube. Both the homogenate and medium were stored at −20°C until steroid extraction. For time course experiments, triplicate dishes (5×10^5 cells/dish) were incubated in the presence of 1 nM T for 1.5, 3, 6, 12, and 24 h, under exactly the same experimental conditions. Medium and cells were therefore processed as described above.

Steroid Extraction. Steroid extraction was carried out on the incubation medium, because we have shown previously it contains proportionally greater amounts of radioactive steroids than those found in the cells (8), as well as on tissue homogenates. Before sample manipulation, all of the glassware was coated with 4 μg of radioinert T to minimize radioactivity losses. Extraction of free steroids was performed on 1-ml aliquots of the incubation medium or 1-ml aliquots of tissue homogenates with 10 ml of diethyl ether. The resulting ether phase (free steroids) was evaporated to dryness under nitrogen stream in a water bath at 37°C. The dried residues were then resuspended in 3 ml of acetone, transferred to test tubes, and dried again in the Speed Vac. The aqueous phase (conjugate steroids) was freeze dried in a SVC100H Speed Vac evaporator concentrator (Savant Instruments, Inc., Farmingdale, NY), resuspended in a solution consisting of 970 μl of acetate buffer (0.2 M, pH 5.0) and 30 μl of Glusulase enzyme mixture (DuPont Co., Wilmington, DE), and incubated for 18 h at 37°C to hydrolyze the steroid conjugates (sulfates and glucuronides). Hydrolyzed steroids were extracted as before using 10 ml of diethyl ether; the resulting aqueous phase was read for nonextracted radioactivity in a β-counter (Beckman Instruments Italia, Milan, Italy), whereas the ether phase was processed as described below. The two ether extracts (free and hydrolyzed steroids) were finally stored at −20°C until chromatographic analysis. The extraction efficiency was calculated as reported previously (6). To separate glucuronide from sulfate steroids, duplicate aliquots of incubation medium or tissue homogenate were extracted with diethyl ether as before. The resulting aqueous phase was acidified using 50 mg of ascorbic acid and laid on Bond Elut-C18 cartridge (Waters) in a Vac-Elut vacuum system SPS24 (Analytichem Int., Harbor City, CA). Cartridges were rinsed once with 3 ml of water:methanol (95:5, v/v), and conjugate steroids were differentially eluted using 2 ml of water:methanol (60:40, v/v) for sulfates and 3 ml of methanol for glucuronides. Aliquots (500 μl) of both fractions were read in a β-counter and estimated as percentage values over the total radioactivity detected.

Chromatographic Analysis. Extracted steroids were chromatographically analyzed in the reverse phase mode (reverse phase-HPLC), using a Beckman model 324 HPLC system equipped with a UV detector (model 160), set at 280 nm, and an on-line Flo-One/β (500TR Series) three-channel Flow Scintillation Analyzer (Packard Instrument Co., Meriden, CT). Steroids were separated under isocratic condition using an Ultrasphere octadecyl silane (Beckman, Fullerton, CA) column (250 × 4.6 I.D. mm) and an optimized mobile phase consisting of acetonitrile:tetrahydrofuran:0.05 M citric acid (38:6:56, v/v/v) at a flow rate of 1 ml/min. Radiometric detection was performed using a 1-ml flow cell and an Ultima-Flo-M (Camberra-Packard) scintillation mixture at a flow rate of 4 ml/min. Routine data integration was achieved by the Flo-one Radio-HPLC workstation software package (Packard) and computed in net cpm, after correction for both residence time and background subtraction (40 cpm for tritium).

RT-PCR Analysis.

RNA isolation. Total RNA were isolated from cells or tissue samples using the one step method described by Chomczymski (9). All of the RNAs were treated with RNase-free DNase I to get rid of the minimum contamination of genomic DNA. The extracted amounts of RNA were determined by measuring the absorbance at 260 nm, and the RNA integrity was assessed by nondenaturating agarose gel electrophoresis.

RT-PCR. The semiquantitative analysis was performed using the Titan one tube RT-PCR system (Roche Molecular Biochemicals, Indianapolis, IN). All samples were first analyzed using β-actin primers (5′-AGG AGC ACC CCG TGC TGC TGA-3′ as forward primer and 5′-CTA GAA GCA TTT GCG GTG GAC-3′ as reverse primer), and the RNA levels for aromatase analysis were normalized based on the results obtained from β-actin analysis. RT-PCR conditions were as follows. After 1-h incubation at 50°C, 25 cycles of PCR were run at 94°C for 30 s, 50°C for 1 min, and 68°C for 2 min. The reaction was ended with a 7-min elongation period at 68°C. We have shown previously that, using these 25 cycles of RT-PCR, PCR products accumulate exponentially, and their quantity increases in an mRNA-dependent manner (10). In addition, control PCR analyses of RNA without the reverse transcriptase reaction were performed to ensure that potentially contaminating DNA could not serve as a template for amplification. The RT-PCR was performed using two oligonucleotides with sequences derived from exon 2 region of the human aromatase gene: 5′-GAC TCT AAA TTG CCC CCT CTG-3′ (forward primer) and 5′-GTG CCC TCA TAA TTC CAC AC-3′ (reverse primer).

Hybridization. The RT-PCR products (10 μl each) were run on 1.5% agarose gel, transferred onto the positively charged ζ membrane (Bio-Rad Laboratories, Hercules, CA) in 20 × SSC, and UV cross-linked. The membranes were hybridized with DIG-11–ddUTP-labeled probe, using the 3′-end oligonucleotide labeling kit (Roche Molecular Biochemicals), with a sequence (5′-ATG GTT TTG GAA ATG CTG AA-3′) derived from the region in between the two primers used for PCR reaction. Hybridization using a third probe further ensured that the PCR products were those expected. The blots were prehybridized in DIG-Easy-Hyb (Roche Molecular Biochemicals) for 1 h and hybridized overnight at different hybridization temperatures with different probes. The hybridization temperature was generally 5°C to 10°C below probe's melting temperature. After hybridization, the blots were washed twice for 1 min in 2 × SSC, 0.1% SDS and twice for 15 min in 0.1 × SSC, 0.1% SDS.

Chemiluminescent Detection. The RT-PCR products were detected according to the standard procedure. After hybridization and washes, membranes were rinsed briefly in washing buffer (maleic acid buffer). The membranes were incubated for 30 min in blocking solution, then in DIG-AP (1:10000) solution for 30 min, two times for 15 min in washing buffer, and equilibrated 2 min in detection buffer. The CSPD ready-to-use solution was applied on the membranes that were incubated 5 min at room temperature. After incubation for 1 min at 37°C, the membranes were exposed to Lumi-film Chemiluminescent detection film (Roche Molecular Biochemicals) for 15–30 min at room temperature. Image density was quantified using an Imaging Densitometer (Model GS-670; Bio-Rad Laboratories).

RESULTS

Androgen Metabolism. The CL and HepG2 cell lines exhibited strikingly divergent patterns of androgen metabolism using either labeled T or Ad as precursor. A 24-h incubation of HepG2 cells with 1 nM T resulted in fact in a massive degradation of the precursor, the proportion of T remaining below 4%; metabolic products were represented by Ad (~30%) and estrogens (mostly estrone), the latter accounting for >50% of all radioactive metabolites. It is worth mentioning that both estrone (E1) and estradiol (E2) were present as conjugates, mainly sulfates. Results after 72-h incubation were even more clear cut, with estrogens representing nearly the totality of radioactivity detected (Fig. 1*A*). Equivalent results were observed using Ad as precursor (Fig. 1*B*). In contrast, nonneoplastic CL cells did not show any detectable aromatase activity, using either T or Ad as precursor, at any incubation time. However, incubation of this cell line with T gave rise to remarkable amounts of DHT and 3α/3β-androstanediols (3α/3β-diols; ~22% and 42% as a sum by 24 and 72 h, respectively), whereas the proportion of 17keto androgens (including Ad and its 5α-reduced derivative androstanedione, 5α-A) did not exceed 7% by 72-h incubation (Fig. 1*C*). Similar results were obtained after incubation with Ad, although a reduced formation of

DHT and 3α/3β-diols (≤23% as a sum) and a much greater proportion of 17keto androgens (~75%, including 5α-A, androsterone, and its epimer epiandrosterone) were observed in this cell line (Fig. 1*D*).

Interestingly, the use of increasing T concentrations resulted in a dose-dependent decrease of estrogen formation, with a corresponding increase of 17keto androgen production (Fig. 2). In this respect, although the majority of T metabolic products in HepG2 cells was represented by estrogen sulfates, the proportion of free derivatives raised significantly using 100 nM T as precursor (Fig. 2). Absolute estimates (as total fmoles) of T metabolic course in HepG2 cells revealed a nonlinear behavior in the formation of both estrogens and unconjugated products, suggesting that aromatase and sulfotransferase enzyme activities may be reduced at higher T doses (data not shown).

As indicated by time course experiments (Fig. 3), estrogen formation occurred early in HepG2 cells, being respectively >10 and 20% after only 3- and 6-h incubation with T. This was accompanied by a proportional decrease of unconverted T precursor (28 and 10%) and parallel rise in the extent of Ad and 17keto androgen formation (59 and 66%). After 12- and 24-h incubation, HepG2 cells displayed an additional increase of estrogen formation (38 and 67%, respectively), the majority being represented by E1 sulfate (59% by 24 h). Conversely, Ad formation dropped to 53 and 19%, respectively, after 12- and 24-h incubation.

Notably, a 24-h exposure of HepG2 cells to the aromatase inhibitor LZ resulted in a striking decrease of estrogen formation from T, which became virtually absent at 0.4 nM LZ. In particular, LZ showed an IC_{50} dose for aromatase inhibition as low as 0.2 nM (Fig. 4).

Patterns of androgen metabolism and rates of estrogen formation were also inspected in both tissue minces (Fig. 5*A*) and the resulting primary epithelial and stromal cell cultures (Fig. 5*B*). Studies on tissue

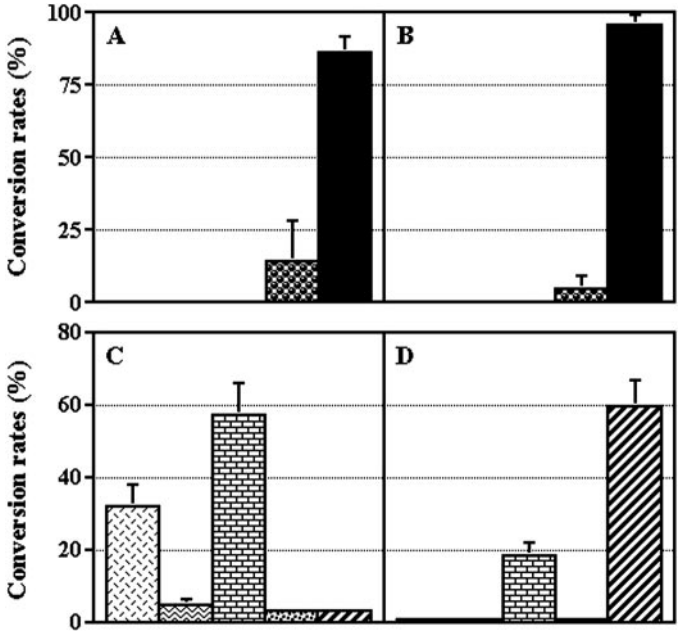

Fig. 1. Androgen metabolism in malignant HepG2 cells (*A* and *B*) and nontumoral CL (*C* and *D*) cell lines after 72-h incubation with labeled T (*A* and *C*) or Ad (*B* and *D*) as precursor. Data represent mean ± SD values of radioactivity detected from triplicate experiments. T; DHT; 3α/3β-diols; Ad; 17keto; E2-S; E1-S.

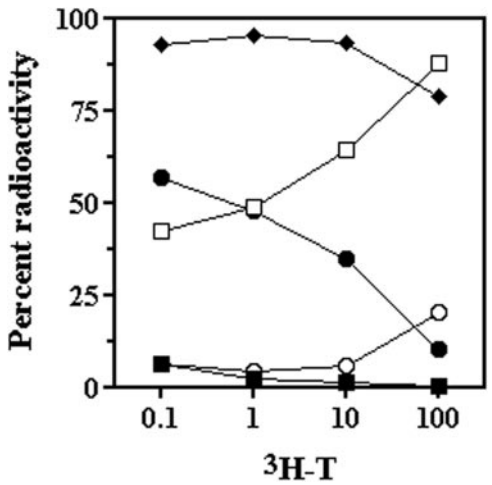

Fig. 2. Patterns of androgen metabolism in HepG2 cells using increasing precursor (*T*) concentrations. ■, T; □, 17keto; ●, estrogens; ○, free; ♦, conjugates.

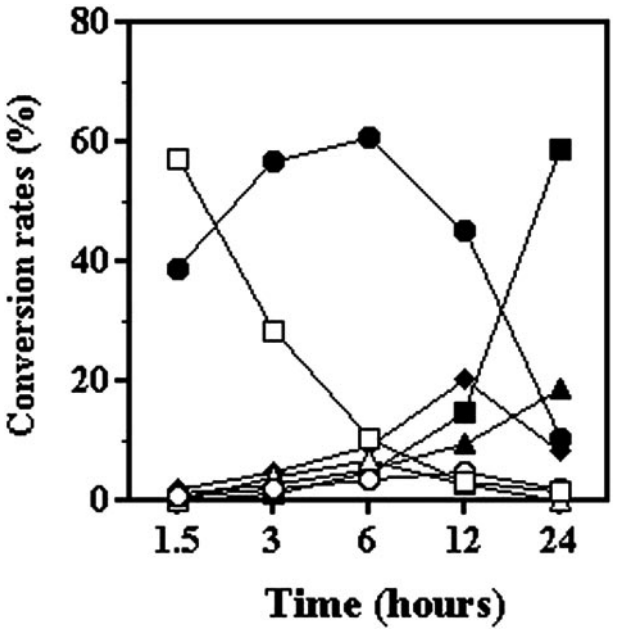

Fig. 3. Time course of T metabolism in HepG2 hepatoma cells. □, T; ○, DHT; ●, Ad; △, E2; ♦, E1; ■, E1-S; ▲, 17keto.

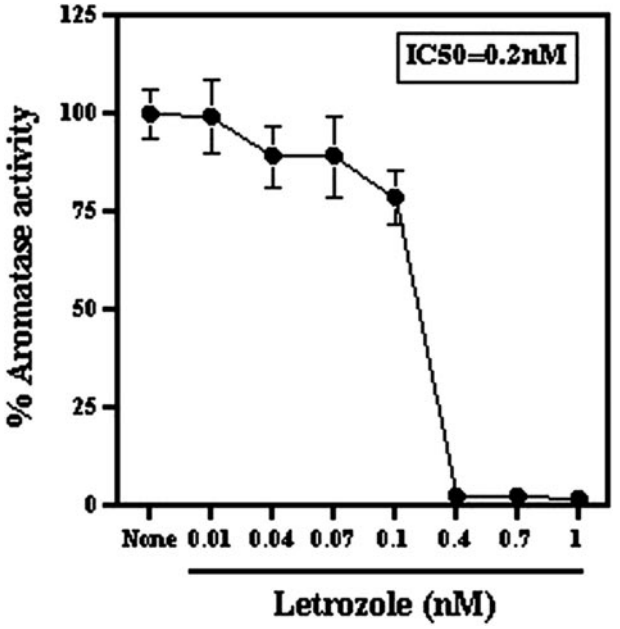

Fig. 4. Effects of the aromatase inhibitor LZ on aromatase activity in HepG2 cells.

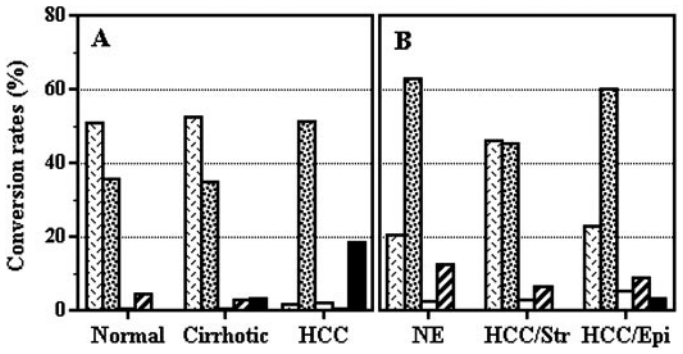

Fig. 5. metabolism in normal, cirrhotic, and HCC tissues (*A*) and primary cultures of nontumoral epithelial (*NE*), HCC stromal (*HCC/Str*), and HCC epithelial (*HCC/Epi*) liver cells (*B*). Either minced tissues (*A*) or primary cell cultures (*B*) were incubated for 24 h with labeled T, as described in "Materials and Methods." ⊠ T; □ 5α,17Red; ▩ Ad; ▨ 17keto; ■ estrogens.

minces revealed that no estrogen formation could be detected in normal liver tissue, whereas it attained ~3.5% in cirrhotic samples and ~20% in HCC tissues after a 24-h incubation with T. Interestingly, androgen metabolic pathways were very similar in both normal and cirrhotic specimens, with equivalent rates of T conversion (~50%) and Ad formation (35%). By contrast, HCC tissues, likewise HepG2 cells, showed a massive degradation of T (>97%) and a greater Ad production (>50%). Results from T metabolism in primary epithelial and stromal cell cultures originated from the very same tissue samples indicate that aromatase activity could be detected only in HCC epithelium, although at a lower extent (3.2%), with respect to the whole tissue. No estrogen formation was observed in both normal hepatocytes and HCC stromal cells. Surprisingly, T metabolism was quite similar in normal liver and HCC epithelial cells, except for estrogen formation, with comparable rates of both T conversion (~80%) and Ad production (~60%) after 24-h incubation. On the other hand, HCC stromal cells showed lower (54%) conversion rates of T and proportionally reduced amounts of Ad (45%) and 17keto (6%) androgen derivatives.

RT-PCR Analysis. Results of RT-PCR agree with those obtained from chromatographic analysis of aromatase activity in both tissues and cells. Aromatase mRNA was detected in both CL and HepG2 cell lines using the RT-PCR method. The RT-PCR was performed in quadruplicate. The level of aromatase mRNA in the HepG2 cell line was determined to be 2.3 times greater than that observed in CL cells (Fig. 6). Exon-specific RT-PCR analysis revealed that aromatase mRNA in CL cells is mainly exon I.3A-containing message, whereas in HepG2 cells, it is predominantly exon I.4-containing message. RT-PCR analysis of aromatase expression in tissue samples revealed significantly lower levels in nontumoral liver with respect to HCC tissue samples. Cirrhotic tissues exhibited, however, variable expression levels, which were in turn comparable with those detected in nonneoplastic or HCC specimens (Fig. 6).

DISCUSSION

We report here consistent evidence that human HCC tissues and HepG2 hepatoma cells contain elevated aromatase activity, as opposed, respectively, to nontumoral hepatic tissues and nonmalignant CL cells, where no aromatase activity could be detected using our intact cell analysis. To our knowledge, this is the first report by which activity of the aromatase enzyme is strikingly different in nontumoral, cirrhotic, and malignant human liver tissues and cells, respectively, being undetectable, variable, and elevated. Although a few previous studies (11, 12) have found high aromatase activity in primary and metastatic tumors of human liver, there is some inconsistency in the expression of aromatase enzyme in the surrounding hepatic tissue (13); still other groups have failed to detect an increase of aromatase expression in hepatoma tissues (14). In our work, consistently greater aromatase expression and activity have been revealed in HCC tissues and cells, respectively, using RT-PCR method and intact cell analysis, this latter being featured by high reproducibility and low sensitivity limits (5).

As matter of facts, we have observed that HCC tissue minces give rise to ~20% conversion of T into estrogen after 24-h incubation. However, separate primary epithelial and stromal cell cultures, originated for the same HCC tissue samples, indicated that only epithelial cells are endowed with the aromatase enzyme, although its activity is >5-fold lower than that found in the whole HCC tissues. This apparent inconsistency may simply be ascribed to the limited number of HCC epithelial cells in primary cultures with respect to tissue minces, or it may be the result of a potential paracrine stimulatory effect of stromal cells on the aromatase expression and/or activity in epithelial cells, or both.

We have observed that, in HepG2 cells, the majority (>95%) of androgen metabolic products is represented by estrogens, notably estrone, mostly in their sulfate forms. Interestingly, a striking prevalence of 17βhydroxysteroid dehydrogenase (17βHSD) oxidative activity, leading to high conversion rates of either T to Ad or E2 to E1, was also observed in this cell line. By contrast, CL cells exhibited a remarkable formation of both DHT and, especially, its derivatives 3α/3β-androstanediols (3α/3β-diols), suggesting that both the 5α-reductase and 3α/3β-hydroxysteroid dehydrogenase enzyme activities are largely prevalent (Fig. 4). This evidence confirms recent studies indicating that human HCC tissues contain both steroid sulfotransferase and type 2 (oxidative) 17βHSD enzyme activities (15). In our study, time course experiments clearly reveal that HepG2 cells have high 17βHSD, aromatase, and sulfotransferase activities and that these enzymes act in an orderly, strictly regulated succession, eventually leading to a nearly complete transformation of androgen precursor (T or Ad) into estrogen sulfates.

We have also investigated aromatase expression in nontumoral, cirrhotic, and malignant human liver tissues and cells by RT-PCR analysis. The results obtained indicate that normal liver tissues and

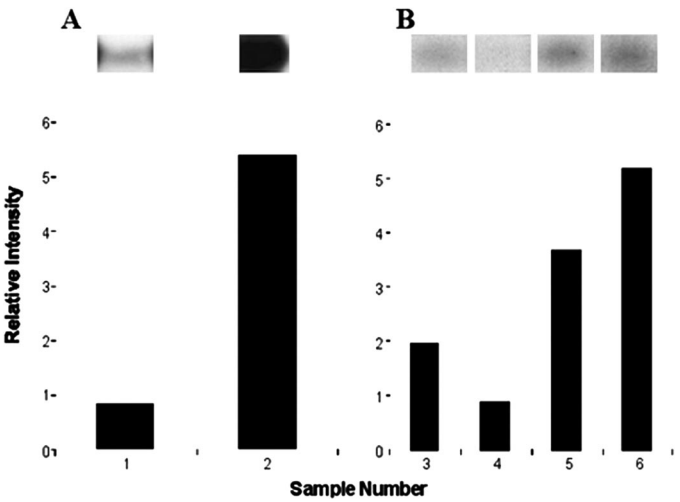

Fig. 6. RT-PCR analysis of aromatase expression in liver cell lines and liver tissues. *A* and *B*, results obtained from cell lines and liver tissue, respectively. The original images of the RT-PCR products are shown at the *top* of each *panel*. The analyses were performed with CL cells (*Lane 1*), HepG2 hepatoma cells (*Lane 2*), nontumoral liver tissue (*Lane 3*), cirrhotic liver tissue 1 (*Lane 4*), cirrhotic liver tissue 2 (*Lane 5*), and HCC tissue (*Lane 6*). The analysis was performed in quadruplicate with the procedures described in "Materials and Methods."

cells express significantly lower levels of aromatase, whereas malignant HCC tissues and hepatoma cells have consistently greater amounts of aromatase mRNA. As far as cirrhotic tissue samples are concerned, aromatase expression is in turn comparable with that found in nontumoral or malignant tissues. This evidence suggests that differential aromatase expression in cirrhotic liver, leading to a diverse local estrogen accumulation, may be responsible for different potentialities of cirrhotic tissues to develop HCC.

Previous reports have suggested that excessive aromatase activity in HCC tissues is caused by transcriptional transactivation of the P450 aromatase gene via the proximally located promoters I.3 and II (16). Our data indicate that, although aromatase activity was not detectable in CL cells, a low level of aromatase mRNA expression was observed by RT-PCR, however. Results from exon-specific RT-PCR analysis suggest that aromatase mRNA in CL cells is mainly exon I.3A-containing message, different from HepG2 cells that have predominantly exon I.4-containing message. Recent studies by Okubo *et al.* (17) indicate that exon I.3A-containing message has a low translation efficiency. Therefore, the aromatase protein in CL cells may be very low, thus explaining the results from aromatase activity measurement.

In the literature, there is indirect but converging evidence that steroid hormones, especially estrogens, may play an important role in the etiology of human HCC. In particular, it has been proposed that an imbalance of serum T and E2, with a decrease in the T:E2 ratio, may promote the development of HCC from cirrhosis (18). In addition, ERs have been reported to represent a major prognostic factor for survival of HCC patients (19). Nonetheless, several randomized clinical trials on the use of tamoxifen to improve the survival of advanced HCC patients have produced thus far negative results, regardless of the ER status of tumor tissues (20). On the basis of both *in vivo* and *in vitro* data, different independent groups have hypothesized that tamoxifen action in HCC is mediated by an ER-independent pathway (4, 21). An additional explanation may be provided by the occurrence of variant ER, especially in male HBsAg-positive patients. In this respect, Villa *et al.* (22) have proposed that the presence of ER variants at an early stage of liver disease, such as chronic active hepatitis, may represent a prominent mechanism facilitating neoplastic transformation. Studies on the impact of antiandrogens and/or chemical castration on the prognosis of HCC patients have failed to produce any significant improvement of patients' survival because of either lack of activity or excessive toxicity (23). Overall, there is no convincing evidence that endocrine treatment may favorably affect the outcome of HCC patients.

In the present work, we report clear evidence that aromatase activity and expression is elevated in HCC tissues and cells and that it may represent an important factor for development of HCC from cirrhosis. In a previous study, Gunson *et al.* (24) have revealed that fadrozole, a nonsteroidal aromatase inhibitor, significantly reduces the incidence of spontaneous HCC in both male and female Sprague Dawley rats. This combined evidence would imply that local estrogen formation from androgens might play a role in the development and/or maintenance and progression of human HCC. This may provide a basis to improve endocrine treatment of HCC patients using alternative strategies with antiaromatase agents.

ACKNOWLEDGMENTS

The aromatase inhibitor Letrozole was kindly provided by Novartis International AG, Basel, Switzerland.

REFERENCES

1. WHO World Health Report WHO. Geneva: WHO, 1997.
2. Nagasue, N., Ito, A., Yukaya, H., and Ogawa, Y. Estrogen receptors in hepatocellular carcinoma. Cancer, *57:* 87–91, 1986.
3. Villa, E., Camellini, L., Dugani, A., Zucchi, F., Grottola, A., Merighi, A., Buttafoco, P., Losi, L., and Manenti, F. Variant estrogen receptor messenger RNA species detected in human primary hepatocellular carcinoma. Cancer Res., *55:* 498–500, 1995.
4. Barkhem, T., Andersson-Ross, C., Hoglund, M., and Nilsson, S. Characterization of the "estrogenicity" of tamoxifen and raloxifene in HepG2 cells: regulation of gene expression from an ERE controlled reporter vector versus regulation of the endogenous SHBG and PS2 genes. J. Steroid Biochem. Mol. Biol., *62:* 53–64, 1997.
5. Castagnetta, L., Granata, O. M., Lo Casto, M., Calabrò, M., Arcuri, F., and Carruba, G. Simple approach to measure metabolic pathways of steroids in living cells. J. Chromatogr., *572:* 25–39, 1991.
6. Castagnetta, L., Granata, O. M., Polito, L., Blasi, L., Cannella, S., and Carruba, G. Different conversion metabolic rates of testosterone are associated to hormone-sensitive status and -response of human prostate cancer cells. J. Steroid Biochem., *49:* 351–357, 1994.
7. Castagnetta, L., Granata, O. M., Bellavia, V., Amodio, R., Scaccianoce, E., Notarbartolo, M., Follari, M. R., Miceli, M. D., and Carruba, G. Product of aromatase activity in intact LNCaP and MCF-7 human cancer cells. J. Steroid Biochem. Mol. Biol., *61:* 287–292, 1997.
8. Castagnetta, L., Granata, O. M., Lo Casto, M., Miserendino, V., Calò, M., and Carruba, G. Estrone conversion rates by human endometrial cancer cell lines. J. Steroid Biochem., *25:* 803–809, 1986.
9. Chomczymski, P. *In:* F. Ausubel, R. Brent, R. Kingston, D. Moore, J. Seidmen, J. Smith, and K. Struhl (eds.), Current Protocols in Molecular Biology, Vol. 1, pp. 4.2.1–4.2.2. New York: John Wiley & Sons Inc., 1998.
10. Zhou, C., Zhou, D., Esteban, J., Murai, J., Siiteri, P., Wilczynski, S., and Chen, S. Aromatase gene expression and its exon I usage in human breast tumors. Detection of aromatase messenger RNA by reverse transcription-polymerase chain reaction. J. Steroid Biochem. Mol. Biol., *59:* 163–171, 1996.
11. Bulun, S., Noble, L., Takayama, K., Michael, M., Agarwal, V., Fisher, C., Zhao, Y., Hinshelwood, M., Ito, Y., and Simpson, E. Endocrine disorders associated with inappropriately high aromatase expression. J. Steroid Biochem. Mol. Biol., *61:* 133–139, 1997.
12. Harada, N., Ota, H., Yoshimura, N., Katsuyama, T., and Takagi, Y. Localized aberrant expression of cytochrome P450 aromatase in primary and metastatic malignant tumors of human liver. J. Clin. Endocrinol. Metab., *83:* 697–702, 1998.
13. Yabuuchi, I., Kawata, S., Tamura, S., Ito, N., Matsuda, Y., Nishioka, M., Moriwaki, K., Matsuzawa, Y., and Tarui, S. Aromatase activity in human hepatocellular carcinoma. Relationship with degree of histologic differentiation. Cancer, *71:* 56–61, 1993.
14. Yamamoto, T., Sakai, C., Yamaki, J., Takamori, K., Yoshiji, S., Kitawaki, J., Fujii, M., Yasuda, J., Honjo, H., and Okada, H. Estrogen biosynthesis in human liver–a comparison of aromatase activity for C-19 steroids in fetal liver, adult liver and hepatoma tissues of human subjects. Endocrinol. Jpn., *31:* 277–281, 1984.
15. Narasaka, T., Moriya, T., Endoh, M., Suzuki, T., Shizawa, S., Mizokami, Y., Matsuoka, T., and Sasano, H. 17β-hydroxysteroid dehydrogenase type 2 and dehydroepiandrosterone in the human liver. Endocrinol. Jpn., *47:* 697–705, 2000.
16. Agarwal, V., Takayama, K., Wyk, J. V., Sasano, H., Simpson, E., and Bulun, S. Molecular basis of severe gynecomastia associated with aromatase expression in a fibrolamellar hepatocellular carcinoma. J. Clin. Endocrinol. Metab., *83:* 1797–1800, 1998.
17. Okubo, T., Truong, T., Yu, B., Itoh, T., Zhao, J., Grube, B., Zhou, D., and Chen, S. Down-regulation of promoter I. 3 activity of the human aromatase gene in breast tissue by zinc-finger protein, Snail (SnaH). Cancer Res., *61:* 1338–1346, 2001.
18. Tanaka, K., Sakai, H., Hashizume, M., and Hirohata, T. Serum testosterone:estradiol ratio and the development of hepatocellular carcinoma among male cirrhotic patients. Cancer Res., *60:* 5106–5110, 2000.
19. Villa, E., Moles, A., Ferretti, I., Buttafoco, P., Grottola, A., Buono, M. D., Santis, M. D., and Manenti, F. Natural history of inoperable hepatocellular carcinoma: estrogen receptors' status in the tumor is the strongest prognostic factor for survival. Hepatology, *32:* 233–238, 2000.
20. Liu, C., Fan, S., Ng, I., Lo, C., Poon, R., and Wong, J. Treatment of advanced hepatocellular carcinoma with tamoxifen and the correlation with expression of hormone receptors: a prospective randomized study. Am. J. Gastroenterol., *95:* 218–222, 2000.
21. Tan, C., Chow, P., Findlay, M., Wong, C., and Machin, D. Use of tamoxifen in hepatocellular carcinoma: a review and paradigm shift. J. Gastroenterol. Hepatol., *15:* 725–729, 2000.
22. Villa, E., Dugani, A., Moles, A., Camellini, L., Grottola, A., Buttafoco, P., Merighi, A., Ferretti, I., Esposito, P., Miglioli, L., Bagni, A., Troisi, R., Hemptinne, B. D., Praet, M., Callea, F., and Manenti, F. Variant liver estrogen receptor transcripts already occur at an early stage of chronic liver disease. Hepatology, *27:* 983–988, 1998.
23. Pignata, S., Daniele, B., Gallo, C., Vivo, R. D., Monfardini, S., and Perrone, F. Endocrine treatment of hepatocellular carcinoma. Any evidence of benefit? Eur. J. Cancer Clin. Oncol., *34:* 25–32, 1998.
24. Gunson, D., Steele, R., and Chau, R. Prevention of spontaneous tumors in female rats by fadrozole hydrochloride, an aromatase inhibitor. Br. J. Cancer, *72:* 72–75, 1995.