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, Shiuam Chen, and Giuseppe Carruba

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 Leetrozole resulted in a striking decrease of estrogen formation, which became virtually absent at a Leetrozole dose of 0.4 μM. 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 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. 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, different clinical trials have investigated the potential impact of antiestrogens (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, or to alternative, nonreceptor 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% CO₂ 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 cholelithiasis. 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 10–15 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 × 10⁵ 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 μM labeled T ([1,2,6,7-3H(N)]-T, specific activity

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1 To whom requests for reprints should be addressed, at Experimental Oncology, Department of Clinical Oncology, ARNAS-Civico, Via Paravaccino 143, 90127 Palermo, Italy. E-mail: lucasblb@umipa.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, Leetrozole; RT-PCR, reverse transcription-PCR.
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 TG C TG CTA-3′ as forward primer and 5′-CTA GAA GAA TTG 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. 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 oligonucleotide probes with sequences derived from exon 2 region of the human aromatase gene: 5′-GAC TCT AAA TTG CCC CCT GTG-3′ (forward primer) and 5′-GGT CCC TAA TTT 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 χ membrane (Bio-Rad Laboratories, Hercules, CA) in 20 × SSC, and UV cross-linked. The membranes were hybridized with DIG-11-dUTP-labeled probe, using the 3′-end oligonucleotide labeling kit (Roche Molecular Biochemicals), with a sequence (5′-ATG GTT GG A ATG CTG AA-3′) derived from the region in between the two primers used for PCR reaction. Hybridization using a 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 1°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. 1A). Equivalent results were observed using Ad as precursor (Fig. 1B). 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-androstenediols (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 androstanediene, 5α-A) did not exceed 7% by 72-h incubation (Fig. 1C). 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. 1D).

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. 5A) and the resulting primary epithelial and stromal cell cultures (Fig. 5B). Studies on tissue
As a result of the aromatase enzyme expression in the surrounding hepatic tissue, there is some inconsistency in the expression of aromatase enzyme in the metastatic tumors of human liver. 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.

As a matter of fact, 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 17β-estradiol and estrone, mostly in their sulfate forms. Interestingly, a striking prevalence of 17β-hydroxysteroid dehydrogenase enzyme activities, leading to high conversion rates of either T to E2 or E1 to E2, was also observed in this cell line. By contrast, CL cells exhibited a remarkable formation of both DHT and even more of its derivatives 3α/3β-androstenediols (3α/3β-diols), suggesting that both the 5α-reductase and 3α/3β-hydroxysteroid dehydrogenase enzyme activities are largely prevalent. This evidence confirms recent studies indicating that human HCC tissues contain both steroid sulfotransferase and type 2 (oxidative) 17βHSD enzyme activities. 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

**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
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 different aromatase expression in cirrhotic liver, leading to a diverse local estrogen accumulation, may be responsible for different potencies 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 the 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.

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