Journal of Steroid Biochemistry & Molecular Biology 113 (2009) 290–295

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Androgen metabolism and biotransformation in *nontumoral* and malignant human liver tissues and cells

Orazia M. Granata [a], Letizia Cocciadifero [a], Ildegarda Campisi [a], Vitale Miceli [a], Giuseppe Montalto [b], Lucia M. Polito [a], Biagio Agostara [c], Giuseppe Carruba [a,*]

[a] *Experimental Oncology, Department of Oncology, "M. Ascoli" Cancer Hospital Center, ARNAS-Civico, Palermo, Italy*
[b] *Department of Clinical Medicine and Emerging Pathologies, University of Palermo, Italy*
[c] *Clinical Oncology, Department of Oncology, "M. Ascoli" Cancer Hospital Center, ARNAS-Civico, Palermo, Italy*

A R T I C L E   I N F O

Article history:
Received 28 November 2008
Received in revised form 16 January 2009
Accepted 19 January 2009

Keywords:
Metabolism
Androgen
Aromatase
Estrogen
Normal liver
Cirrhosis
HCC

A B S T R A C T

There is indirect multiple evidence that hints at a potential role of sex steroids in development and progression of human hepatocellular carcinoma (HCC). In the present study, we have investigated androgen metabolism in a panel of human liver cancer cell lines (HA22T, Huh7, HepG2) and in *normal*, cirrhotic and malignant human liver tissues aiming to dissect the potential impact of individual enzyme activities and their products in normal and diseased human liver, both *in vivo* and *in vitro*. Using our *intact cell* analysis we were able to assess rates and pathways of androgen metabolism in living conditions. Overall, incubation of cultured cells or tissue minces with either testosterone (T) or androstenedione (Ad) used as precursor resulted in a large extent of 17βoxidation of T to Ad (cells: 28–77%; tissues: 35–50%). In malignant liver cell lines, both HA22T and Huh7 cells showed consistent amounts of the 5α-reductase enzyme products (18% and 15%, respectively), while 5β-reductase activity was more pronounced in Huh7 cells (18%) than in HA22T cells (1.8%). Interestingly, a significant extent of estrogen formation could be observed in Huh7 cells (5.4–11.5%), while no aromatase activity could be detected in HA22T cells. In HepG2 cells, along with a relatively high proportion of Ad, estrogens represented the most prominent (50–55%) end product of androgen metabolism, regardless of the precursor used. In liver tissues, equivalent results could be obtained, with a consistent proportion of 17βoxidation of T to Ad (35–50%) being observed in the majority of samples. However, while normal liver tissue samples exhibited a minor proportion of bioactive androgens (3.4%) with no aromatase products, HCC tissues showed a significant extent of aromatase activity (nearly 20%) with estrogen representing the most prominent metabolic product after 24 h incubation with either T or Ad. HCV and alcoholic cirrhotic tissues displayed different patterns of androgen metabolism. The former produced limited amounts of bioactive androgens (5.3%) and considerable levels of the intermediate aromatase product 19OH-Ad (up to 28%), the latter exhibited a prevalence of androgen degradation through the 5β-reductase pathway (9.8%) and a significant extent of aromatase activity (16% as a whole). In conclusion, three major metabolic states could be depicted, depending on prevalent pathways of androgen metabolism and steroid receptor status: *estrogenic*, *androgenic*, and *mixed*. This model supports the idea that local estrogen biosynthesis may be implicated in human HCC and provides a basis for the exploitation of aromatase inhibitors and/or ER antagonists or selective estrogen receptor modulators (SERMs) as a new therapeutic strategy in HCC patients.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Primary hepatocellular carcinoma (HCC) represents the fifth leading cancer and the third most common cause of cancer death worldwide [1]. In Italy, mortality rates have been drastically increasing from 4.8 deaths/100.000 in 1969 up to 11.0/100.000 in 1994, with even higher values in the South of Italy (Italian ISTAT database).

The most clearly established risk factors for HCC are chronic infections with hepatitis B virus (HBV) or hepatitis C virus (HCV), cirrhosis, alcohol abuse, exposure to dietary aflatoxin and male sex [1]. Independent of race and geography, HCC incidence in men is in fact two to three times higher than 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. Clinical observations and death statistics

* Corresponding author at: Experimental Oncology, Department of Oncology, ARNAS-Civico, Piazzale N. Leotta 2, 90127 Palermo, Italy. Tel.: +39 091 66 64348; fax: +39 091 66 64352.
E-mail address: lucashbl@unipa.it (G. Carruba).

0960-0760/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2009.01.013

have indicated that chronic viral hepatitis B and C progress more rapidly in males that in females, and that cirrhosis is largely a disease of men and postmenopausal women [2]. On the other hand, a positive association between circulating testosterone and risk of HCC has been reported [3]. In addition, there is consistent evidence that androgen receptor (AR) levels are significantly higher in malignant than in *nontumoral* human liver [4,5]. Based on this combined evidence, human HCC has been considered an androgen-dependent tumor.

Previous evidence revealed that estrogen receptors (ER) are expressed in primary HCC [6]. Two major ER types exist, the classical ERα and the recently discovered ERβ [7]. Their balance appears to be important to determine the biological impact of estrogen in target tissues and cells. Some study suggests that ERβ is an antagonist of ERα-regulated gene transcription in the mice liver [8]. Moreover, studies in human liver tissues to assess the clinical significance of ERα and ERβ have indicated that both receptors are expressed at higher levels in patients with chronic liver disease compared with those with HCC and that an excess of the variant ERδ5 is associated with wild-type ERβ in patients with severe liver disease and/or HCC [9].

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 [10,11]. This, however, could be partly due to the presence of mutant ERα forms, as previously described [12]. Recently, the use of synthetic progestins (megestrol) for treatment of patients having inoperable HCC characterized by mutant liver ER resulted in a significant increase of median survival of patients [13].

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 17β-hydroxysteroid dehydrogenases, 5α-reductase, and aromatase. A diverse expression and/or activity of these enzymes may eventually lead to a differential accumulation of more or less biologically active steroids in liver tissues and, therefore, may affect differently proliferative activity of hepatocytes.

We have previously reported that both expression and activity of the aromatase enzyme, that converts androgen into estrogen, is elevated in malignant human liver tissues and cells, as opposed to *nontumoral* hepatic tissues and *nonmalignant* liver cells where it remains undetectable [14]. Furthermore, we have recently indicated that metabolic patterns of androgens may diverge substantially in different human malignant liver cell model systems [15].

In this study, we have assessed the activity of key enzymes governing metabolic pathways of androgens, including aromatase, 17β- and 3α-hydroxysteroid dehydrogenases (HSD), 5α- and 5β-reductase, in a panel of malignant human liver cell lines and in *normal*, cirrhotic and HCC human liver tissues. This aiming to dissect the contribution of individual enzyme activities to metabolic profiles of androgens and, hence, their potential biological impact in normal and diseased human liver, both *in vivo* and *in vitro*.

2. Materials and methods

2.1. Cell cultures

The HepG2 and Huh7 cells (ATCC, Rockville, MD) and HA22T/VGH (generously provided by Prof. M. Levrero, Rome Italy) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum and L-glutamine (2 mM) and DMEM medium supplemented with 10% fetal calf serum and sodium pyruvate (1 mM) L-glutamine (2 mM), respectively, and grown in a humidified 5% CO_2 atmosphere in air at 37 °C.

Cells having a narrow range of passage number were used for all experiments. To determine rates and direction of androgen metabolism, cells (5×10^5 cells/dish) were seeded in 30 mm Petri dishes and processed as described below.

2.2. Tissue samples

Tissues of HCC (n 8), and of either HCV (n 6) or alcoholic (n 4) cirrhosis, were obtained from patients undergoing liver surgical resection. Histologically normal liver tissues (n 5) were also obtained from patients during surgery for benign liver disease (e.g. colelithiasis). Written informed consent was obtained in all cases. Fresh tissue samples were collected at the surgical theatre and immediately transported to cell culture unit in 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 resuspended in fresh medium and seeded onto 30 mm Petri dishes to assess steroid metabolism (see below).

2.3. Androgen metabolism

The methods and procedures used to assess metabolic rates and pathways of sex steroids (*intact cell* analysis) have been originally established and optimized in our laboratories [16–18]. Briefly, cell or tissue cultures were washed twice with phosphate-buffered saline (PBS)-A and incubated for either 24 or 72 h in FCS-free, phenol red-free RPMI medium containing 1 nM tritiated androgen ([1,2,6,7-^{3}H(N)]-testosterone, S.A. 92.4 Ci/mmol, or [1,2,6,7-^{3}H(N)]-androstenedione, S.A. 84.5 Ci/mmol; DuPont de Nemours Italiana SpA, Milan, Italy) as precursor. For cultured cells, following incubation medium was transferred to plastic tubes and stored at −80 °C until analysis. As far as tissue cultures are concerned, minced tissues were resuspended in the incubation medium, transferred to a plastic tube and centrifuged at 2000 rpm for 5 minutes. The resulting pellet was gently homogenized using a glass-glass Dounce homogenizer (Kontes Co., Vineland, NJ) in 3 ml of PBS, while the supernatant (incubation medium) was transferred to a separate tube. Both the homogenate and the medium were stored at −80 °C until steroid extraction. Medium and cells were therefore processed as described below.

2.4. Steroid extraction and chromatographic analysis

Steroid extraction was carried out on the incubation medium, since we have previously shown it contains proportionally greater amounts of radioactive steroids than those found in either cell or tissue homogenates [19]. Extraction of steroids was performed with SPE method in Vac-Elut apparatus using C18 cartridges on 1 ml aliquots of medium, as previously described [20]. Briefly, two fractions were collected: in the first, conjugate (sulfate and glucuronide) steroids were eluted using water–methanol solution (60:40, v/v); in the second, the free (unbound) steroids were eluted using water–methanol solution (15–85, v/v). The two fractions were dried in a SVC100H Speed Vac evaporator-concentrator (Savant Instruments Inc, Farmigdale, NY) and conjugate steroids were hydrolized at 37 °C for 18 h, in 1 ml of a solution consisting of 970 μl of 0.2 M acetate buffer (pH 5.0) and 30 μl of Glusulase enzyme mixture (duPont Co, Wilmingon, DE). The hydrolized steroids were extracted again with SPE method using ethylacetate and evaporated to dryness, as described above. Both free and conjugate steroids were analyzed in RP-HPLC using a Beckman 324 model HPLC system equipped with an UV detector set at 280 nm, and an *on line* Flo-One/*beta* (500TR Series) three-channel

Table 1
Metabolic conversion rates of androgens in malignant human liver cells.

	HA22T/VGH		Huh7		HepG2	
	T	Ad	T	Ad	T	Ad
T	3.3	–	4.2	4.4	4.0	2.3
Ad	77.0	81.5	52.7	55.3	27.6	31.7
DHT	–	–	9.2	8.8	–	–
5αAdione	14.7	15.7	–	–	–	–
EpiA	1.0	1.1	2.3	1.5	–	–
A	2.2	1.7	1.3	–	–	–
3αEtio	–	–	17.5	18.6	13.3	15.6
3βEtio	1.8	–	–	–	–	–
19OH-Ad	–	–	7.3	–	3.2	3.0
E2	–	–	0.5	–	–	–
E1	–	–	1.0	–	–	–
E2-S	–	–	–	0.7	7.8	2.9
E1-S	–	–	3.9	10.8	44.0	44.5

Cells were incubated 24 h in the presence of either testosterone (T) or androstenedione (Ad) used as precursor and metabolic conversion rates determined as described in Section 2; results represent average conversion rates from triplicate experiments; abbreviations: T, testosterone; Ad, androstenedione; DHT, dihydrotestosterone; 5αAdione, 5α-androstanedione; 3α/3βdiol, 3α/3β-androstanediol; Epi-A, epiandrosterone; A, androsterone; 3αEtio, etiocholan-3α-ol-17-one; 3βEtio, etiocholan-3β-ol-17-one; 19OH-Adione, 19hydroxy-androstenedione; E2, estradiol; E1, estrone; E2-S, estradiol-sulfate; E1-S, estrone-sulfate.

flow scintillation analyzer (Packard Instrument Co, Meriden, CT). Steroids were eluted under isocratic condition using a Ultrasphere ODS column (250 × 4.6 I.D. mm) and an optimized mobile phase consisting of acetonitrile: tetrahydrofuran: 0.05 M citric acid (39: 6: 55, v/v/v) at a flow rate of 1 ml/min. Radiometric detection was performed using a 1 ml flow cell and Ultima-Flo-M (Camberra-Packard) scintillation mixture at 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.

3. Results

In this work we have assessed rates and direction of androgen metabolism in cultured human liver cancer cells and in *nontumoral*, cirrhotic and malignant human liver tissues.

3.1. Androgen metabolism in malignant human liver cell lines

Overall, data from our *in vitro* studies reveal that activities of steroid enzymes, including aromatase, sulfotransferase, 17βHSD, 5α- and 5β-reductases, are markedly divergent in malignant HepG2, Huh7 and HA22T/VGH human liver cell lines.

Results of 24 h incubation of cells with either testosterone (T) or androstenedione (Ad) as androgen precursor are reported in Table 1.

In the first place, all the three cell lines showed a remarkable extent of androgen metabolism, with on average only 3.6% of either precursor remaining unconverted after incubation. Secondly, results of 24 h incubation with T and Ad were strictly comparable in all cell lines.

Both HA22T and Huh7 cells showed a largely prevalent oxidative metabolism, the Ad being the predominant androgen metabolite after 24 incubation with either T (HA22T: 77%; Huh7: 53%) or the same Ad (HA22T: 82%; Huh7: 55%). Also 5α-reductase activity was comparable in the two cell lines, accounting on average for 18% and 12% of all androgen metabolites, respectively. However, while 5αAdione was the major 5α-reduced androgen in HA22T cells (over 15%), Huh7 cells produced significant amounts (around 9%) of the biologically active androgen DHT. This finding was equivalent using either T or Ad as precursor. Furthermore, products of the 5β-reductase pathway were present in both cells lines, although to a much lower extent in HA22T cells (3βEtio: 1.8%) than in Huh7 cells (3αEtio: 18% as a mean). More importantly, Huh7 cells exhibited a remarkable extent of aromatase activity (12.7% and 11.5% using T and Ad as precursor, respectively), while aromatase could not be detected in HA22T cells. Interestingly, Huh7 cells incubated 24 h with T displayed 5.4% of estrogen formation, while the remaining part of aromatase products (7.3%) was represented by the intermediate aromatase metabolite, the 19OH-Ad. Conversely, when Huh7 cells were incubated with Ad, the totality of aromatase products was represented by estrogens, notably estrone, in their sulfated forms (11.5%).

As far as HepG2 cells are concerned, the extent of 17βoxidation, leading to Ad accumulation, was apparently lower than in the other two cell lines, with rates of Ad ranging from 28% to 32% after 24 h incubation with T or Ad, respectively. In addition, 5β-reductase pathway eventually led to formation of 3αEtio (respectively 13% and 16% using T and Ad as precursor), while no 5α-reductase activity could be detected. However, aromatase was by far the most prominent enzyme activity in this cell line, with aromatase products representing 55% and 50% of all radioactive metabolites respectively using T and Ad as precursor. With the exception of a 3% formation of the intermediate metabolite 19OH-Ad, the vast majority of aromatase products were represented by estrogens and, likewise in Huh7 cells, by estrogen (notably estrone) sulfate. This pattern was independent of the precursor used.

Major pathways of androgen metabolism *in vitro* are illustrated in Fig. 1. As it can be seen, 5α-reductase (20%) was the most prominent enzyme activity in HA22T cells, being 10 times and 6 times greater than 5β-reductase and 3αHSD, respectively. The Huh7 cells showed a more balanced picture, with 3αHSD activity being greater than 5β-reductase, while an equivalent extent (nearly 13%) of 5β-reductase and aromatase could be detected in this cell line. Conversely, aromatase activity (over 50%) was largely prevalent (3.9-fold greater) over both 5β-reductase and 3αHSD in HepG2 cells.

3.2. Androgen metabolism in human liver tissues

Patterns of androgen metabolism were also investigated in minced liver tissue samples, under exactly the same experimental conditions used for *in vitro* studies.

These studies were conducted on a series of *normal*, cirrhotic (HCV and alcoholic), and malignant human liver tissues. As reported in Table 2, all tissues exhibited a consistent proportion of T oxidation into Ad, with a range of 35–50%. Despite the considerable

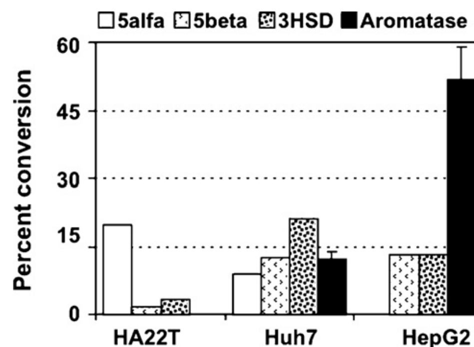

Fig. 1. Pathways of androgen metabolism in malignant human liver cell lines. Cells were incubated for 24 h with tritiated T as precursor and metabolic conversion rates assessed as described in Section 2. Data represent mean ±SD (error bars) of percent conversion rates from triplicate experiments conducted in duplicate.

Table 2
Metabolic conversion rates of androgen in *nontumoral*, cirrhotic and malignant human liver tissues.

Tissue	Radioactive metabolite												
	T	Ad	DHT	3α/βdiols	5αAdione	EpiA	A	Etio	3αEtio	3βEtio	19OH-Ad	E2	E1
Normal (n 5)	44.01 ±8.24	35.45 ±7.45	0.64 ±0.83	2.74 ±0.28	ND	6.02 ±2.37	1.12 ±0.33	2.54 ±0.23	11.78 ±2.63	1.87 ±0.21	ND	ND	ND
HCV Cirrhosis (n 6)	16.77 ±7.23	49.11 ±17.23	0.97 ±0.41	4.33 ±0.76	1.30 ±0.39	2.95 ±0.73	0.89 ±0.42	1.63 ±0.21	9.82 ±5.74	1.91 ±0.82	28.19 ±21.17	ND	ND
Alcoholic Cirrhosis (n 4)	30.39 ±18.47	49.26 ±17.35	ND	ND	0.88 ±0.09	1.14 ±0.67	0.57 ±0.09	1.93 ±0.54	5.57 ±2.22	2.29 ±1.27	11.86 ±10.44	2.77 ±0.72	1.43 ±1.12
HCC (n 8)	39.99 ±19.26	44.14 ±11.29	1.36 ±0.66	0.60 ±0.07	1.34 ±0.29	2.03 ±1.49	3.99 ±2.19	0.71 ±0.38	2.95 ±1.24	1.49 ±0.76	ND	4.22 ±1.19	15.45 ±4.35

Tissue minces were incubated 24 h in the presence of testosterone (T) used as precursor and metabolic conversion rates determined as described in Section 2; results represent conversion rates (mean ± SD) from triplicate experiments; abbreviations: *Normal*, healthy liver; HCV, hepatitis C virus; HCC, hepatocellular carcinoma, T, testosterone; Ad, androstenedione; DHT, dihydrotestosterone; 3α/3βdiol, 3α/3β-androstanediol; 5αAdione, 5α-androstanedione; Epi-A, epiandrosterone; A, androsterone; Etio, etiocholanolone; 3αEtio, etiocholan-3α-ol-17-one; 3βEtio, etiocholan-3β-ol-17-one; 19OH-Ad, 19hydroxy-androstenedione; E2, estradiol; E1, estrone.

extent (35%) of 17βoxidation of T to Ad, *normal* liver tissue samples did not exhibit detectable amounts of either estrogen or intermediate products (19OH-Ad) of the aromatase enzyme. Overall, in these tissues, formation of biologically active androgens, including DHT and 3α/3βdiols, was limited (3.3% as a sum), while androgen degradation through the 5β-reductase pathway accounted for more than 16% as a whole. In HCV-cirrhotic tissues, similar profiles of androgen metabolism could be observed, with a limited proportion (around 5%) of bioactive androgen and a slightly lower extent of 5β-reductase (13%) being seen at 24 h. Interestingly, a remarkable formation (over 28%) of the intermediate aromatase product, the 19OH-Ad, was observed, but no estrogen production could be detected. Conversely, in alcoholic cirrhosis tissues, no detectable amounts of bioactive androgens could be found and, overall, major androgen metabolic pathways, including both 5α- and 5β-reductase, were markedly lower than in *normal* and HCV liver tissues. On the other hand, aromatase products consisted of both 19OH-Ad (nearly 12%) and estrogen (estradiol and estrone: 4.2% as a sum) and they represented over 16% of all radioactive metabolites. In HCC tissues, the production of bioactive androgens and 5α-reductase activity were in the range of that observed in both *normal* and HCV tissues. In contrast, 5β-reductase was 1.9, 2.6, and 3.1 times lower than that measured in alcoholic cirrhosis, HCV cirrhosis, and in *normal* tissue samples, respectively. More importantly, a significant extent of estrogen formation (nearly 20% as a sum) was observed, while no detectable amounts of 19OH-Ad could be revealed.

Overall, major androgen enzyme activities were significantly different among normal, cirrhotic and malignant liver tissues. As illustrated in Fig. 2, *normal* liver and HCV-cirrhosis showed comparable profiles of androgen metabolism, with the 3αHSD being the leading enzyme activity (22% and 18%, respectively). In both conditions, the respective extent of 5α-reductase, 5β-reductase, and 3αHSD was in the same order of magnitude (namely 5α < 5β < 3αHSD), while no aromatase activity could be detected.

A significantly lower proportion of 3αHSD activity was observed in alcoholic cirrhosis (7.3%) and HCC (9.6%), along with a progressively lower extent of 5β-reductase (9.8% in alcoholic cirrhosis; 5.2% in HCC). As far as aromatase is concerned, estrogen formation was observed in both alcoholic cirrhosis (4.2%) and, to a larger extent (19.7%), in HCC tissues.

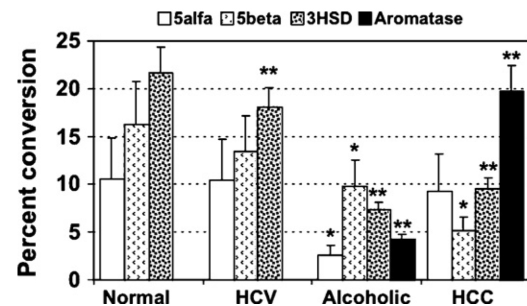

Fig. 2. Pathways of androgen metabolism in *nontumoral*, cirrhotic, and malignant human liver tissues. Rates and patterns of androgen metabolism were assessed in liver tissue minces under exactly the same experimental conditions used for *in vitro* studies (see Section 2). Data represent mean ±SD (error bars) of percent conversion rates from triplicate experiments conducted in duplicate. Normal, healthy liver; HCV, hepatitis C virus cirrhosis; alcoholic, alcoholic cirrhosis; HCC, hepatocellular carcinoma. $^{*}p < 0.05$; $^{**}p < 0.01$.

4. Discussion

In spite local metabolism may dictate the ultimate biological impact of sex steroids in peripheral target tissues and cells, studies on androgen metabolism in either normal or malignant human liver are surprisingly rare. In the present study we have assessed rates and patterns of androgen metabolism in different liver cancer cell lines and in *normal*, cirrhotic and malignant human liver tissues, using our *intact cell* analysis that permits the simultaneous measurement of several steroid enzyme activities in living conditions. The major objective of this study was to determine the potential association of different androgen metabolic pathways with individual cell and/or tissue model systems.

In the first place, comparable data were obtained *in vitro* and *in vivo*. Androgen precursors were actively metabolized, with a remarkable extent of 17βHSD oxidative activity being observed especially *in vitro* but also *in vivo*. The 17βoxidation of testosterone to androstenedione ranged in fact from 35% to 50% *in vivo* and from 28% up to 77% *in vitro*. A moderate production of biologically relevant androgens (DHT, 3αdiols) could be observed in both normal and HCV-cirrhotic liver tissues, while it was undetectable or negligible in alcoholic cirrhosis or HCC tissues, respectively. The enzymatic

control of intratissue levels of bioactive androgens, apart from the 5α-reductase enzyme, is mostly a consequence of the type 1 3αHSD, found exclusively in the liver, and the more widely expressed type 3 3αHSD enzymes (AKR1C4 and AKR1C2, respectively) [21]. The significantly higher 3αHSD activity we detected in normal liver tissues as compared to cirrhosis and, especially, to HCC supports the concept that formation of bioactive androgens is associated with cell differentiation and inversely related to liver injury and damage.

On the other hand, the 5β-reductase enzyme activity, that governs the production of inactive androgen derivatives, consistently decreased from normal liver to cirrhotic and HCC tissue samples, with an inverse relationship with aromatase activity and estrogen formation. It is important to note that the 5β-reductase enzyme can negatively regulate the intracellular concentrations of other bioactive steroid hormones, including progesterone and, to a lesser extent, mineralcorticoids and glucocorticoids [22]. The significant decrease of 5β-reductase activity observed in alcoholic cirrhosis and HCC tissues may also result in liver injury due to the accumulation the cytotoxic intermediate metabolite 7-α-hydroxy-4-cholesten-3-one in bile acid synthesis [23].

The evidence that alcoholic cirrhosis is associated with a decrease of bioactive androgen production and a corresponding appearance of aromatase activity and estrogen formation is in agreement with previous studies reporting a significant increase of circulating levels of estrogens either in male alcoholic cirrhotic patients or in postmenopausal women with a higher consumption of alcohol, even in the absence of liver disease [24,25]. Interestingly, no estrogen formation could be observed in the HCV-cirrhosis tissues, in spite of the elevated levels (28%) of the intermediate 19-ol aromatase product. This finding could be a result of the presence of aromatase mutant(s) that are unable to complete the third step (aromatization) of the enzymatic reaction, as previously reported [26].

In conclusion, as depicted in Fig. 3, three major metabolic pathways of androgens could be identified: (a) the 5α-reductase and the 3αHSD pathways, both leading to the formation of biologically relevant metabolites including DHT and 3αdiols, respectively; (b) the 5β-pathway, governing androgen degradation to inactive derivatives; and (c) the aromatase pathway, responsible for local

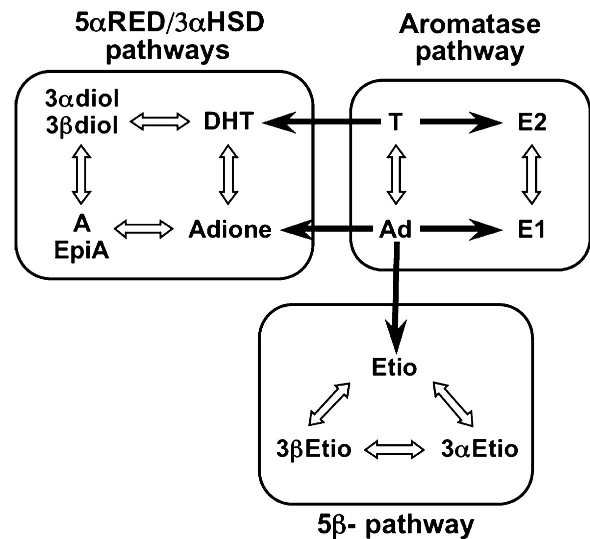

Fig. 3. Major androgen metabolic pathways in human liver tissues and cells. Abbreviations: 5αRED, 5α-reductase; 3αHSD, 3α-hydroxysteroid dehydrogenase; 5β, 5β-reductase. DHT, dihydrotestosterone; 3α/3βdiol, 3α/3β-androstanediol; A, androsterone; Epi-A, epiandrosterone; Adione, 5α-androstanedione; T, testosterone; Ad, androstenedione; E2, estradiol; E1, estrone; Etio, etiocolanolone; 3αEtio, etiocholan-3α-ol-17-one; 3βEtio, etiocholan-3β-ol-17-one.

biosynthesis of estrogens from either testosterone or androstenedione. The extent of individual pathways was markedly diverse comparing malignant cell lines and different liver tissues. Overall, production of biologically relevant androgens, originating from the 5αRED and/or the 3αHSD pathway, is largely dominant in normal and HCV-cirrhotic liver tissues as like as in malignant HA22T cells. Conversely, aromatase-driven estrogen formation is consistently prevalent in HCC tissues and HepG2 hepatoma cells. An intermediate picture could be observed in both alcoholic cirrhosis and Huh7 liver cancer cells, with a more balanced proportion of bioactive androgens and estrogens being formed. Based on this combined evidence, three major metabolic states can be depicted (see Fig. 4). In the *estrogenic* condition (Fig. 4A), aromatase activ-

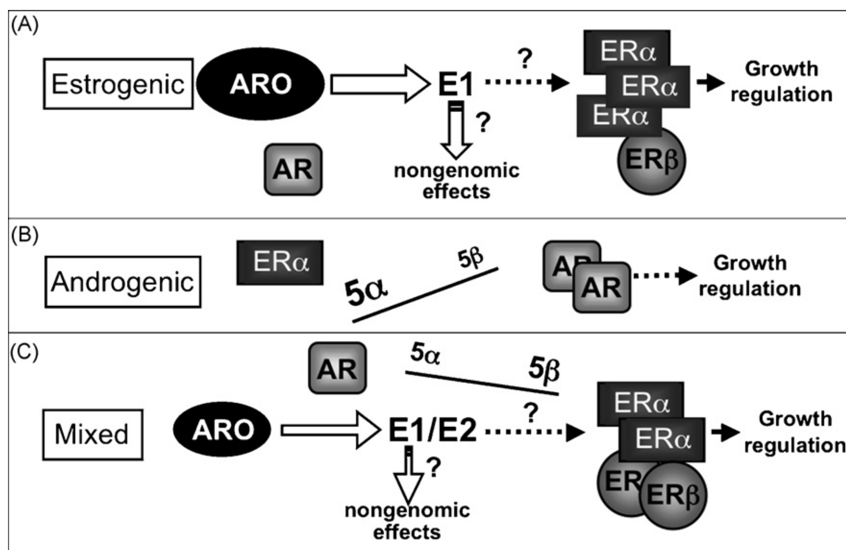

Fig. 4. Major metabolic conditions in human liver tissues and cells: a hypothetical model. (A) *Estrogenic*: aromatase is largely prevalent and the E1 produced may affect liver cell growth through ER-mediated, either genomic or nongenomic, effects. (B) *Androgenic*: no aromatase activity can be detected, while bioactive androgens control cell proliferation through AR-mediated mechanisms. (C) *Mixed*: aromatase activity is yet prevalent, as bioactive androgens are inactivated by the 5β-reductase, with estrogens (E1 and/or E2) being primarily responsible for regulation of liver cell growth. ARO, aromatase; AR; androgen receptor; ER, estrogen receptor; 5α, 5αRED/3αHSD pathway; 5β, 5β-pathway; E2, estradiol; E1, estrone.

ity is largely prevalent and gives rise to locally elevated estrogen (estrone) that may in turn regulate liver cell growth through ER-mediated, either genomic or nongenomic effects. In the *androgenic* condition (Fig. 4B), while aromatase activity is either absent or impaired, the production of bioactive androgens is the predominant metabolic pathway, with androgen derivatives controlling cell proliferation through androgen receptor-mediated mechanisms. In the *mixed* condition (Fig. 4C), aromatase-driven estrogen formation is lower than in the estrogenic state but yet prevalent over biologically relevant androgens as they are being inactivated by a more pronounced 5β-reductase enzyme. In this latter condition, estrogens (estrone and/or estradiol) may primarily be responsible for regulation of liver cell growth through either genomic or nongenomic signaling.

This hypothetical model, while is in accord with our previous studies indicating that aromatase is overexpressed in malignant human liver tissues and cells, it supports the assumption that local estrogen biosynthesis may be important in human HCC development and/or progression. This may provide an experimental basis for the exploitation of selective therapeutic strategies in HCC using aromatase inhibitors and ER antagonists or selective estrogen receptor modulators (SERMs) in both *estrogenic* and *mixed* conditions, and 5α-reductase inhibitors and antiandrogens in the *androgenic* state. In this framework, aromatase expression/activity could be used as a surrogate marker to predict response of HCC patients to endocrine treatment.

References

[1] ACS American Cancer Society, Global cancer: facts and figures (2007).
[2] T. Poyard, V. Ratziu, F. Charlotte, Z. Goodman, J. McHutchinson, J. Albrecht, Rates and risk factors of liver fibrosis progression in patients with chronic hepatitis C, J. Hepatol. 34 (2001) 730–739.
[3] M.W. Yu, C.J. Chen, Elevated serum testosterone levels and risk of hepatocellular carcinoma, Cancer Res. 53 (1993) 790–794.
[4] M.J. Iqbal, M.L. Wilkinson, P.J. Johnson, R. Williams, Sex steroid receptor protein in foetal, adult and malignant human liver tissue, Br. J. Cancer 48 (1983) 791–796.
[5] N. Nagasue, A. Ito, H. Ykaya, Y. Ogawa, Androgen receptors in hepatocellular carcinoma and surrounding parechyma, Gastroenterology 89 (1985) 643–647.
[6] N. Nagasue, A. Ito, H. Yukaya, Y. Ogawa, Estrogen receptors in hepatocellular carcinoma, Cancer 57 (1986) 87–91.
[7] G.G.J.M. Kuiper, E. Enmark, M. Pelto-Huikko, S. Nilsson, J.A. Gustafsson, Cloning a novel estrogen receptor expressed in rat prostate and ovary, Proc. Natl. Acad. Sci. U S A 93 (1996) 5925–5930.
[8] M.K. Lindberg, S. Movérare, S. Skrtic, H. Gao, K. Dahlman-Wright, J. Gustafsson, C. Ohlsson, Estrogen receptor (ER)-β reduces ERα – regulated gene transcription, supporting a “Ying Yang” relationship between ERα and ERβ in mice, Mol. Endocrinol. 17 (2003) 203–208.
[9] M. Iavarone, P. Lampertico, C. Seletti, M.F. Donato, G. Ronchi, E. Del Ninno, M. Colombo, The clinical and pathogenetic significance of estrogen receptor-β expression in chronic liver diseases and liver carcinoma, Cancer 98 (2003) 529–534.
[10] P.F. Engstrom, B. Levin, C.G. Moertel, A. Schutt, A phase II of tamoxifen in hepatocellular carcinoma, Cancer 65 (1990) 2641–2643.
[11] CLIP-Group, Tamoxifen in treatment of hepatocellular carcinoma: a randomized controlled trial. CLIP-Group (Cancer of the liver Italian programme), Lancet 352 (1998) 17–20.
[12] E. Villa, L. Camellini, A. Dugani, F. Zucchi, A. Grottola, A. Merighi, P. Buttafoco, L. Losi, F. Manenti, Variant estrogen receptor messanger RNA species detected in human primary hepatocellular carcinoma, Cancer Res. 55 (1995) 498–500.
[13] E. Villa, I. Ferretti, A. Crottola, P. Buttafoco, M.G. Buono, F. Giannini, M. Manno, H. Bertani, A. Dugani, F. Manenti, Hormonal therapy with megestrol in inoperable hepatocellular carcinoma characterized by variant estrogen receptors: a prospective, randomized, controlled trial, Br. J. Cancer 84 (2001) 881–885.
[14] L.A.M. Castagnetta, B. Agostana, G. Montalto, L. Polito, I. Campisi, A. Saetta, T. Itoh, B. Yu, S. Chen, G. Carruba, Local estrogen formation by nontumoral, cirrhotic, and malignant human liver tissues and cells, Cancer Res. 63 (2003) 5041–5045.
[15] O.M. Granata, L. Cocciadiferro, V. Miceli, L.M. Polito, I. Campisi, G. Carruba, Metabolic profiles of adrogens in malignant human liver cell lines, Ann. N. Y. Acad. Sci. 1089 (2006) 262–267.
[16] L. Castagnetta, O.M. Granata, M. Lo Casto, M. Calabrò, F. Arcuri, G. Carruba, Simple approach to measure metabolic pathways of steroids in living cells, J. Chromatogr. 572 (1991) 25–39.
[17] L. Castagnetta, O.M. Granata, L. Polito, L. Blasi, S. Cannella, G. Carruba, Different conversion metabolic rates of testosterone are associated to hormone-sensitive status and -response of human prostate cancer cells, J. Steroid Biochem. 49 (1994) 351–357.
[18] L. Castagnetta, O.M. Granata, V. Bellavia, R. Amodio, E. Scaccianoce, M. Notarbartolo, M.R. Follari, M.D. Miceli, G. Carruba, Product of aromatase activity in intact LNCaP and MCF-7 human cancer cells, J. Steroid Biochem. Mol. Biol. 61 (1997) 287–292.
[19] L. Castagnetta, O.M. Granata, M. Lo Casto, V. Miserendino, M. Calò, G. Carruba, Estrone conversion rates by human endometrial cancer cell lines, J. Steroid Biochem. 25 (1986) 803–809.
[20] L.A. Castagnetta, G. Carruba, O.M. Granata, R. Stefano, M. Miele, M. Schmidt, M. Cutolo, R. Straub, Increased estrogen formation and estrogen to androgen ratio in the synovial fluid of patients with rheumatoid arthritis, J. Rheumatol. 30 (2003) 2597–2605.
[21] I. Dufort, F. Labrie, V. Luu-The, Human type 1 and 3 3α-Hydroxysteroid dehydrogenase: differential lability and tissue distribution, J. Clin. Endocrinol Metab. 86 (2001) 841–846.
[22] A. Charbonneau, V. Luu-The, Genomic organization of a human 5β-reductase and its pseudogene and substrate selectivity of the expressed enzyme, Biochem. Bioph. Acta 1517 (2001) 228–235.
[23] R.C. Lin, M.J. Fillenwarth, X. Du, Cytotoxic effect of 7alpha-hydroxy-4-cholesten-3-one on HepG2 cells: hypothetical role of acetaldehyde-modified delta4-3-ketosteroid-5beta-reductase (the 37-kd-liver protein) in the pathogenesis of alcoholic liver injury in the rat, Hepatology 27 (1998) 100–107.
[24] J. Guéchot, N. Peignei, F. Ballet, M. Vaubourdolle, J. Giboudeau, R. Poupon, Sex hormone imbalance in male alcoholic cirrhotic patients whit and without hepatocellular carcinoma, Cancer 62 (1988) 760–762.
[25] N.C. Onlande-Moret, P.H.M. Peeters, Y.T. van der Schouw, D.E. Grobbee, C.H. van Gils, Alcohol and endogenous sex steroid levels in postmenopausal women: a cross-sectional study, J. Clin. Endocrinol. Metab. 90 (2005)1414–1419.
[26] Y.C. Kao, K.R. Korzekwa, C.A. Laughton, S. Chen, Evaluation of the mechanism of aromatase cytochrome P450. A site-direct mutagenesis study, Eur. J. Biochem. 268 (2001) 243–251.