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Estrogen synthesis in human colon cancer epithelial cells

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Abstract

Epidemiological and experimental data suggest an involvement of estrogen in the development and progression of colorectal cancer. In order to determine whether local synthesis of estrogen occurred in human colonic cancer cells, two colorectal cancer cell lines, HCT8 and HCT116, were evaluated for gene expression and enzyme activity of cytochrome P450 aromatase. In addition, the effect on aromatase expression of charcoal-stripped fetal calf serum, of quercetin and genistein and of tamoxifen and raloxifene was investigated in both cell lines. RT-PCR analysis revealed that colorectal adenocarcinoma cell lines contain aromatase as a major component. The conversion of [^{3}H]-androstenedione to estrone and labeled water was dose-dependently inhibited by 4-hydroxyandrostenedione and obeyed Michaelis–Menten kinetic with apparent Km values of ~20 nM and V_{max} values of approx. 200 and 500 fmol/mg protein/h for HCT8 and HCT116 cells, respectively. After 24 h incubation, genistein (1 μM) significantly increased aromatase activity in HCT8 cells, with no effect on HCT116 cells. In accord with previous observation in reproductive tissues, quercetin (1 μM) significantly inhibited the enzyme activity in both cell lines. Also tamoxifen (100 nM) acted as inhibitor, while raloxifene (10 nM) decreased the enzyme activity only in HCT116 cells. The aromatase gene expression modulation by these effective agents was consistent with their effects on enzyme activity. These findings demonstrate for the first time that colorectal adenocarcinoma cell lines express aromatase. Interestingly, the enzyme activity was inhibited by quercetin, one major dietary flavonoid, by tamoxifen, a hormonal therapeutic agent for breast cancer, and by raloxifene, used in the prevention of postmenopausal osteoporosis.

1. Introduction

Several lines of evidence suggest a potential role for reproductive hormones in the risk of colorectal cancer (CRC). However, it is still controversial whether sex hormones, in particular estrogen, protect the colon and/or colorectal mucosa against neoplastic growth or are involved in the development of large-bowel cancer [1–4].

The expression of mRNA coding for the classical estrogen receptor (ERα) and for the recently cloned ERβ has been described in human colorectal tissues and cell lines [5–7]. In addition, specific binding of radiolabeled estradiol has been demonstrated in normal and malignant colonic epithelial cells [4,7–9] and, although the physiological significance of colonic ERs remains to be elucidated, the complex hormone-receptor is functional in in vitro studies [7,10,11].

In addition to the classical estrogenic tissues, namely the ovaries, placenta, and adipose tissue, the enzyme complex aromatase/cytocrome P450, which transforms androgenic precursors into estrogenic molecules, is expressed in a large series of human tissues and cell lines [12,13]. These observations suggest that the local conversion of steroid precursors to estrogens could be involved in the physiology and/or physiopathology of non-classical estrogen target tissues.

The experimental design included analyses of the expression of functional aromatase enzyme and the role of food flavonoids and of estrogen agonist/antagonist molecules (SERMs) in the control of aromatase enzymatic activity. Results clearly demonstrate that two human CRC cell lines, namely HCT8 and HCT116

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cells, express mRNA for aromatase and that the conversion of androstenedione to estrogens is modulated by both flavonoids and SERMs.

2. Materials and methods

2.1. Cell culture

HCT8 and HCT116 cells, stabilized cell lines from human colon adenocarcinoma, were obtained from the American Type Cultures Collection (ATCC, Rockville, MD, USA) and were cultured in RPMI-1640 containing 1 mM pyruvic acid Na salt and 10% fetal calf serum (FCS) or McCoy's medium with 10% FCS, respectively. Cells were cultured until confluence, then detached with trypsin/ethylenediamine tetracetic acid (EDTA) solution, and plated at the desired confluence.

2.2. Aromatase activity

The aromatase activity was estimated by determining the incorporation of tritium from [^{3}H]A (1β, 2β-N-[^{3}H] androst-4-ene-3,17-dione) (42 Ci/mmol; 1 Ci = 42 GBq; New England Nuclear, Italy) into water as previously described [13]. Cells were seeded in the appropriate growth medium with or without 10% DCS–FCS in the absence or presence of various stimuli. After 3–48 h, cells were washed, resuspended in fresh medium with or without 10% DCS–FCS, and incubated (1×10^6 cells/well) with [^{3}H]A at 0.5–100 nM concentrations for kinetic studies, or with 2 nM [^{3}H]A for single point determination of enzymatic activity, at 37°C in humidified atmosphere for 6 h, unless otherwise stated. The culture plates were placed on ice for 15 min to condense any water vapor. Cells were then pelleted and the incubation medium was removed. Cells were then washed twice with PBS and assayed for protein content. The incubation medium and the combined saline washes were pushed through a pre-equilibrated Sep-Pak $C_1$8 minicolumn (Water Associates, Italy) into a counting vial. The column was washed with water to remove residual [^{3}H]water into the respective counting vials. After addition of scintillation liquid, the radioactivity was counted. Each experiment was conducted in triplicate. To establish the nonspecific release of [^{3}H]water, duplicate aliquots of [^{3}H]A-supplemented cell-free medium were incubated under each experimental condition and the blank value, which averaged 0.8% of the added radioactivity after 6 h incubation, was subtracted from the amount measured in the experimental incubation. The aromatase activity was expressed as fmol of [^{3}H]A metabolized/mg cell protein/h. Each experiment was conducted in triplicate and was repeated at least two times to ensure that the results were quantitatively reproducible.

To validate the [^{3}H]water release assay, the products of aromatase activity were identified by thin layer chromatography (TLC). After incubation of HCT8 and HCT116 cells (1×10^6 cells/well) with 2 nM [^{3}H]A for 12 h, conditioned media were extracted with ether (1:2). After 24 h at −20° C the extracted steroids in ether were separated from the frozen aqueous layer and evaporated. Approx. 300 nmol of testosterone, androstenedione, estrone and 17β-estradiol, used as standards, and triplicate samples reconstituted in dichloromethane were applied to TLC plates and run in dichloromethane-ethyl acetate-methanol (80:20:1). After localization by reference to the mobility of the standards under UV light, the products were scraped from the plates, extracted from the silica gel with ethyl acetate and counted for radioactivity.

2.3. RT-PCR

RNA enriched in polyA$^+$RNA was directly obtained from 2×10^6 HCT8 and HCT116 cells using a commercial kit (mRNA isolation kit, Boehringer Mannheim, Milan, Italy). Aromatase and β-actin (internal standard) mRNAs were retrotranscribed and amplified using a pair of appropriate oligonucleotides as primers (Table 1). The RT-PCR was carried out in a 50 μl reaction volume with the one-tube-two-enzyme system 'Access RT-PCR System' (Promega Corporation, Madison, WI, USA) containing 50 pmol of each primer, 0.5 mM $MgSO_4$, 0.2 mM of each dNTP, 5 units of AMV reverse transcriptase and 5 units of Tfl DNA polymerase. Reverse transcription was performed at 48°C and amplification at 60°C of annealing, according to the manufacturer instructions. The transcripts were electrophoresed through a 2% agarose gel and visualized by ethidium bromide staining of the gel.

2.4. Quantification of RT-PCR products

The optimal number of cycles of amplification to allow quantification of aromatase and β-actin genes PCR products was determined using the appropriate pairs of primers (Table 1). The PCR products for each cycle were subjected to 2% agarose gel electrophoresis and the intensity of ethidium bromide fluorescence was automatically measured as previously described [14]. To test the reproducibility and linearity of the data the image analyses from four separate experiments were used. The amount of aromatase mRNA was expressed in arbitrary units (AU), which represented the ratio value between the ethidium bromide fluorescence of aromatase transcript and β-actin transcript after 35 cycles.

Table 1
Primer sequences for PCR of aromatase and β-actin sequences

Amplified fragments♯row	Primers	
	Name	Sequence
Aromatase	sense — exon 9	5′-GAA TAT TGG AAG GAT GCA CAG ACT-3′
	antisense — exon 10	5′-GGG TAA AGA TCA TTT CCA GCA TGT-3′
β-actin	sense — exon 4	5′-CCC AGC ACA ATG AAG ATC AA-3′
	antisense — exon 5	5′-TTT CTG CGC AAG TTA GGT TTT GACAA-3′

2.5. Statistics

Data were expressed as mean ± SD. Statistical differences were analyzed using one way analysis of variance. Significance was adjusted for multiple comparisons of means using Bonferroni's approximation.

3. Results

Aromatase activity was determined in HCT8 and HCT116 cells by measuring [^{3}H]water released from [^{3}H]A. In all experimental conditions the rate of aromatase activity was linear for up to 24 h incubation (data not shown). Therefore, the enzymatic activity was evaluated by incubating [^{3}H]A for 6 h. DCS-FCS markedly increased the enzymatic activity in both cell lines in a dose-dependent fashion, reaching the maximum at 10% concentration with no significant difference at 20% concentration (Fig. 1).

To evaluate the specificity of the assay, CRC cells were incubated with 2 nM [^{3}H]A in the absence or presence of 10 nM-1 μM concentrations of 4-hydroxyandrostendione (4OH-A), a known steroidal aromatase inhibitor [15]. 4OH-A inhibited the aromatase activity with a dose-dependent fashion reaching approximately 80% suppression of the enzyme activity at 1 μM concentration in both cell lines (Fig. 2).

[^{3}H]water released from [^{3}H]A was a function of the labeled substrate, obeying the Michaelis–Menten kinetic in both HCT8 and HCT116 cells (Fig. 3). Different concentrations of [^{3}H]A were added and [^{3}H]water released from the labeled steroid was evaluated after 6 h incubation with 10% DCS-FCS. Representative results from two experiments in both cell lines are shown in Fig. 3. While Km values observed in both cell lines (approx. 20 nM) were similar to those reported in adipose stromal cells [16], the maximal capacity of [^{3}H]A conversion in HCT8 (~200 fmol/mg protein/h) and HCT116 cells (~500 fmol/mg protein/h) was approximately 10- and 4-fold lower than that of adipose tissue [16].

To validate the [^{3}H]water release assay, fractionation of [^{3}H]A into the putative enzymes (i.e. aromatase and 17β-hydroxysteroid dehydrogenase) products released by HCT8 and HCT116 cells into the medium, was identified by TLC. After incubation of HCT8 cells with 2 nM [^{3}H]A, the radioactivity (mean ± SD of two experiments) expressed as percentage of total counts was fractionated among the four steroids as follows: androstenedione (58.9% ± 2); 17β-estradiol (20.2% ± 0.9); estrone (12.6% ± 0.2); testosterone (8% ± 0.8). Similar results were obtained in HCT116 cells.

The effect of different agents on the conversion of 2 nM [^{3}H]A into [^{3}H]water was evaluated in both cell lines (Fig. 4). In these cells 1 mM Bt_2-cAMP and 100 nM of dexamethasone, known inducers of aromatase activity in adipose cells [12,16], did not modify [^{3}H]A conversion either in FCS-free medium or in FCS-medium. 17β-estradiol (E_2) was used at physiological concentrations (1 nM). Tamoxifen reduced HCT8 and HCT116 cell viability after 24 h incubation at pharma-

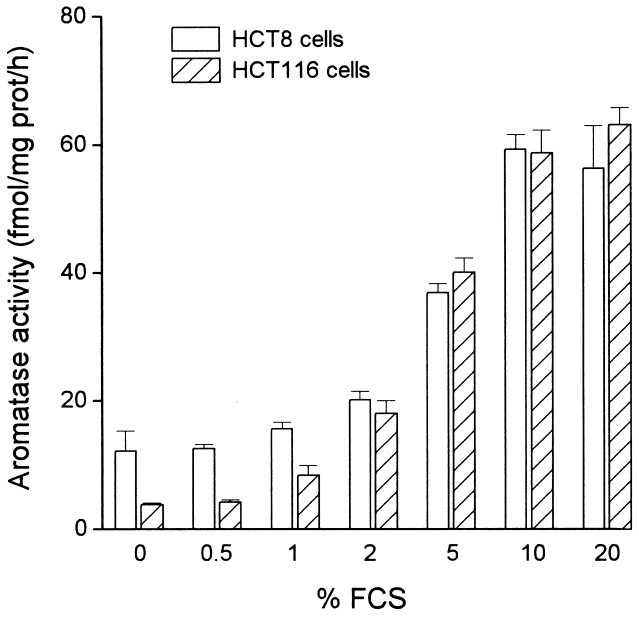

Fig. 1. Effect of DCS–FCS on aromatase activity. After incubation of HCT8 and HCT116 cells with 2 nM [^{3}H]A in the absence or presence of different concentrations (%) of DCS-FCS, aromatase activity was evaluated. Results are expressed as mean ± SD of triplicate from two separate experiments with both cell lines.

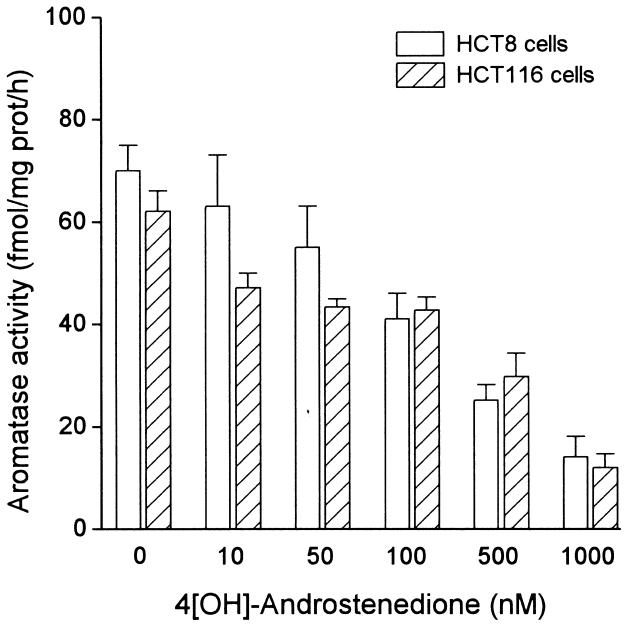

Fig. 2. Inhibition of aromatase activity by 4OH-A. After incubation in FCS-medium with 2 nM [^{3}H]A in the absence or presence of different concentrations of 4OH-A, HCT8 and HCT116 cells were evaluated for aromatase activity. Results are expressed as mean ± SD of triplicate from two separate experiments with both cell lines.

cological concentrations (1 μM). Thus, 10 nM and 100 nM of the agent were used. Conversely, raloxifene, quercetin and genistein were tested at 1 μM concentration. In FCS-free medium HCT8 cells showed a significant reduction of aromatase activity only after 24 h incubation with 100 nM tamoxifen. Conversely, E_2, tamoxifen at both concentrations, and raloxifene significantly inhibited [^{3}H]A conversion in HCT116 cells. In FCS-medium the inhibitory effect of 100 nM tamoxifen was observed in both cell lines, while raloxifene acted as inhibitor only on HCT116 cells. The bioflavonoid quercetin induced a significant reduction of the enzymatic activity in both cell lines, while the isoflavonoid genistein significantly increased androstenedione conversion only in HCT8 cells.

The agents effective on the enzymatic activity were evaluated for aromatase gene expression modulation by RT-PCR analysis. The optimal number of cycles of amplification to allow a semi-quantitation of aromatase and β-actin genes PCR products was determined using the respective pairs of primers reported in Table 1. A linear increase in the signal was obtained between 34 and 37 cycles of amplification for aromatase and β-actin (Fig. 5). Therefore, 35 cycles were used in all subsequent experiments. The aromatase product of the expected size (293 bp) was observed in untreated HCT8 and HCT116 cells (Fig. 6). Treatment of both cell lines with 10% of DCS-FCS induced an increase of the aromatase RT-PCR product while the agents acting as inhibitors of the enzymatic activity in both cell systems induced a decrease of the aromatase RT-PCR product either in the absence or presence of FCS (Fig. 6). In FCS-medium, genistein induced a slight increase of gene expression in HCT8 cells, consistent with its effect on the enzymatic activity (Fig. 6).

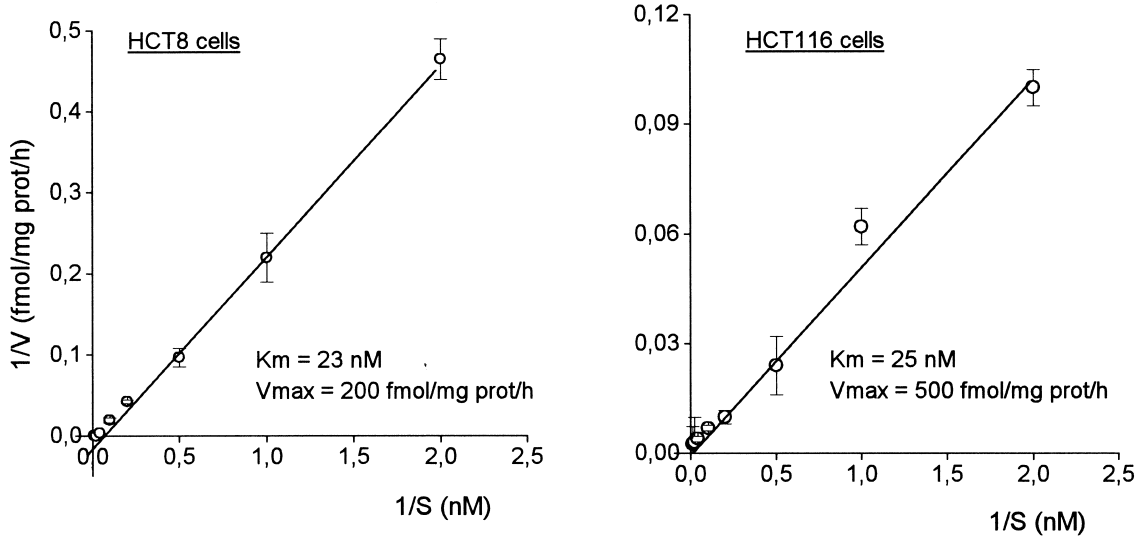

Fig. 3. Lineweaver-Burk plot of [^{3}H]water formed from different concentrations of [^{3}H]A. After 6 h incubation in FCS-medium with [^{3}H]A, HCT8 and HCT116 cells were evaluated for aromatase activity. Results are expressed as mean ± SD of triplicate analysis from two separate experiments.

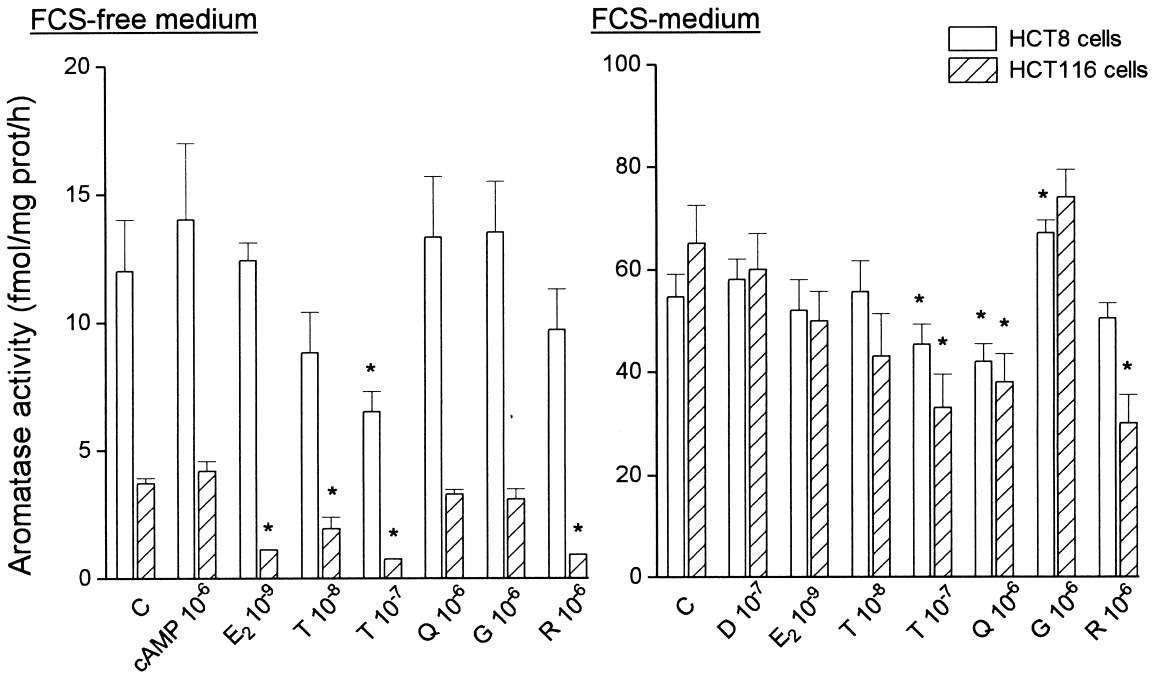

Fig. 4. Effect of different agents on aromatase activity. After 24 h incubation of HCT8 and HCT116 cells with Bt_2-cAMP (cAMP), dexamethasone (D), 17β-estradiol (E_2), tamoxifen (T), quercetin (Q), genistein (G), and raloxifene (R) in the absence or presence of FCS, cells were examined for aromatase activity. Results are expressed as mean ± SD from two or three experiments conducted in triplicate. $^*P < 0.05$ vs control (C).

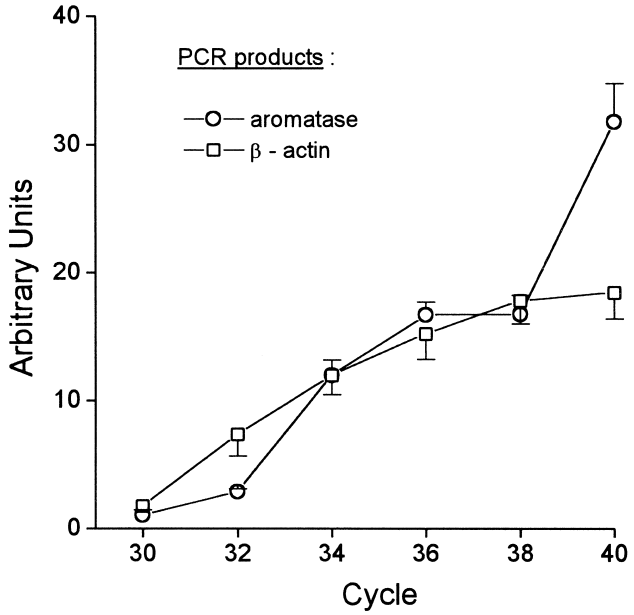

Fig. 5. Determination of the optimal number of amplification cycles for semi-quantification of aromatase expression. Aromatase (○) and β-actin (□) PCR products from mRNA extracts of HCT8 and HCT116 cells were subjected to 2% agarose gel electrophoresis and the intensity of the products from each cycle was measured in arbitrary units. Each point represents the mean ± SD of two experiments conducted with both cell lines.

4. Discussion

Epidemiological studies and in vitro observations suggest that estrogen may directly modulate colonic epithelial cell growth and may be involved in the development of colorectal cancer [1–11]. Estrogen receptors are present in colorectal tissues [4–9] and the complex estrogen/estrogen receptor is functional in in vitro studies [7,10,11]. From in in vivo [6] and in vitro [7] observations it appears ERβ to be the predominant subtype expressed by colorectal epithelial cells. An important variable which regulates estrogen action on colon tissue is, therefore, estrogen receptor expression and function. However, circulating as well as dietary estrogens and consequent estrogenization of colorectal tissue represent another variable in the regulation of cellular responses. Finally, local synthesis of estrogen from androgenic precursors is a third potentially relevant variable.

The enzyme complex aromatase/cytochrome P450, which converts androgenic precursors to estrogens, is widely distributed in gonadal as well as extragonadal tissues [12,13]. Present results clearly show for the first time that human CRC cells also express aromatase cytochrome P450 mRNA and convert androgens to estrogens.

The tritiated water assay used to characterize aromatase activity in CRC cells was validated by a TLC fractionation of [^{3}H]A into the putative enzy-

matic products released by the cells in the medium. In the two cell lines it was observed that the major labeled products were 17β-estradiol and estrone, while testosterone was approx. half of 17β-estradiol. This observation indicated that in CRC cells an active metabolism of androgenic precursors to estrogenic molecules was present and suggested a predominant reductive activity of 17β-hydroxysteroid dehydrogenase.

The conversion of tritiated androstenedione to estrogens and labeled water, occurring in both cell lines, was specific, as demonstrated by the dose-dependent inhibition with the aromatase inhibitor 4OH-A [15] and obeyed the Michaelis–Menten kinetic with Km values similar to that of adipocytes [16]. However, different from other cell systems [12,16], aromatase activity of CRC cell lines was not induced by two classical stimulators of aromatase expression, cAMP and dexamethasone. The molecular mechanisms which underlie this unique behaviour of aromatase regulation in CRC cells in culture needs to be clarified. Interestingly, gene expression and enzyme activity were increased by serum factor(s) and inhibited by estrogenic/antiestrogenic molecules and by quercetin, a widely distributed bioflavonoid.

Evidence is accumulating on the protective role of some phytoestrogens of dietary origin, lignans and isoflavonoids, against breast, prostate, and colorectal cancer [17,18]. Their in vivo and in vitro biological effects appear to be multiple and mediated by different mechanisms of action [17,18]. An inhibitory effect on cell growth by both lignans and isoflavonoids has been reported in human neoplastic cells derived from the gastrointestinal tract [19,20]. Conversely, genistein, a major isoflavone component of soy-based foods, enhances experimental CRC in rats [21]. In addition, some natural and synthetic phytoestrogens have been found to inhibit aromatase/cytochrome P450 in microsomal preparations from human reproductive tissues and in whole adipose cells [22,23]. From the analysis of structure-function relationship of human aromatase it appears that phytoestrogens compete with androgens for the substrate binding site of the enzyme [24].

Present results show a significant inhibition by quercetin on androstenedione conversion in CRC cells, as previously described in microsomal preparations from reproductive tissues [22]. The mechanism by which quercetin inhibits aromatase activity in CRC cells is probably due to a decrease of the enzyme gene expression, even though a direct competition at the substrate binding site of the enzyme can not be ruled out. Conversely, the isoflavone genistein, was not effective in HCT116 cells, as reported in adipose cells [23], while inducing a modest increase of the enzyme activity in HCT8 cells.

SERM molecules also affect aromatase activity. Tamoxifen, a first generation SERM, is, for its antiestrogenic properties, a proven endocrine therapy in es-

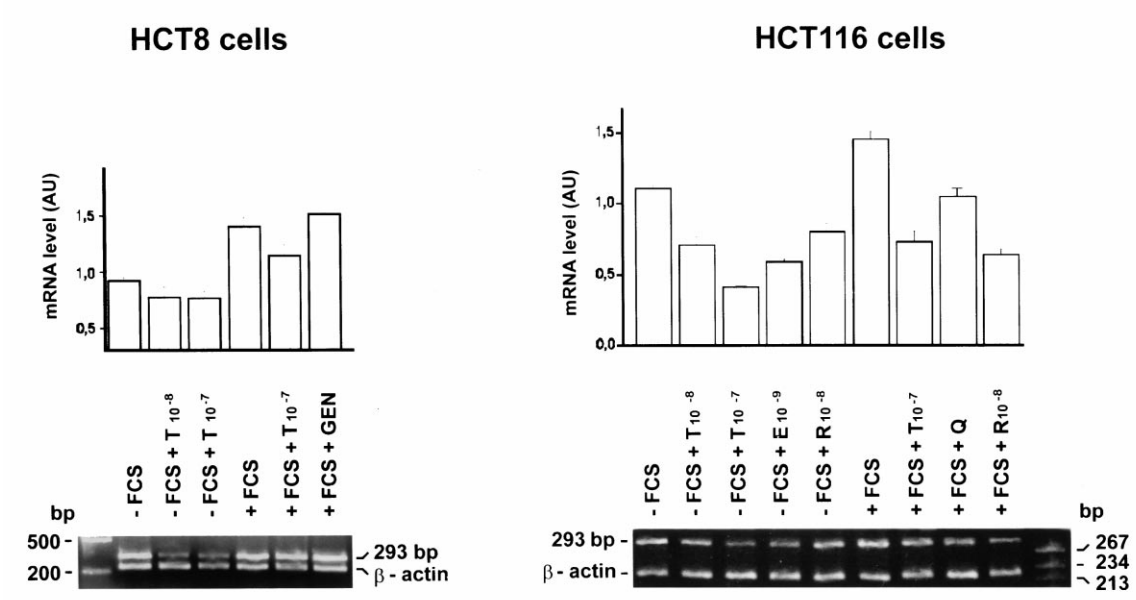

Fig. 6. Effect of different agents on aromatase gene expression in HCT8 and HCT116 cells. RT-PCR for aromatase and β-actin were performed simultaneously on mRNA from cells treated for 24 h with 17β-estradiol (E_2), tamoxifen (T), quercetin (Q), genistein (G), or raloxifene (R) in the absence or presence of 10% FCS. Upper panel: the aromatase PCR transcripts, normalized against β-actin transcripts, were expressed in arbitrary units (AU) as mean ± SD from two different experiments. Lower panel: representative ethidium bromide staining of aromatase (293 bp) and β-actin transcripts. HCT8 and HCT116 cell lines constitutively expressed aromatase mRNA. However, gene expression was modulated by all the enzyme effectors consistently with their action on the enzyme activity.

trogen receptor-positive breast cancer, showing ancillary advantages by acting as estrogen agonist on bone tissue and on lipoprotein metabolism [25]. Raloxifene is a second generation SERM that has estrogen-agonistic effects on bone and cardiovascular systems and estrogen-antagonistic effects on breast and uterus [25]. The in vitro inhibition of CRC cell growth by tamoxifen and by an analog of raloxifene has been previously described [26,27]. In addition, tamoxifen was shown to prevent estradiol proliferative effects on a CRC cell line [11]. These observations, together with the aromatase inhibition here described, suggest that SERMs counteract estrogen effects on CRC tissue by acting as antiestrogens at the receptor level and by lowering the local synthesis of estrogen. For the well-known tissue-specific distribution of gene promoters and enzyme effectors [12,13,16] it could be of interest to investigate whether these drugs modulate the aromatase enzyme in reproductive, adipose, and bone tissues.

It is of interest that molecules such as phytoestrogens and SERMs, which recognize the ERs regulate in different and opposite manner aromatase expression. However, this is not surprising given the recognized multiple mechanisms through which estrogen agonists and antagonists act [28,29]. The relationship between the agonist and antagonist properties of molecules competing for ER binding can, in fact, depend by the expression of ER subtypes, by a repertory of accessory DNA-bound proteins, by co-activators and co-repressors and, perhaps, by non-ER-mediated events [28,29].

In conclusion, the coincident expression of aromatase enzyme, and estrogen receptors in colorectal cancer cells provides the basis for the existence of an autocrine mechanism for estrogen in the neoplastic progression of colon cancer. Even though extrapolation of in vitro data to in vivo conditions should always be critically evaluated, an increased incidence of colon cancer has also been described in conditions of high aromatization, such as in obese subjects [4]. Differences in the enzymatic conversion of androgens towards estrogens among individuals could, therefore, determine a differential tumor progression and spreading. The role of local estrogen synthesis as a function of the tissue-specific microenvironment (i.e. phytoestrogens of dietary origin) on the pathogenesis of colorectal cancer and its potential in the prevention and therapy of this type of tumor requires further investigations.

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