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Abstract. It is well documented that alcohol is associated with an increased risk factor for breast carcinogenesis although the underlying mechanisms are not clearly understood. It has been reported that *in vitro*, the culture of estrogen receptor (ER) expressing breast cancer cells in ethanol containing medium was associated with an increase in the proliferation rate, in the ERα content as well as in ER transcriptional activity. Since these changes are not observed in ER negative breast cancer cells, and since alcohol intake has been associated to an increased level of circulating estrogens, we have postulated that aromatase expression could be increased following ethanol exposure. The results of our studies show a 1.3-fold increase in cell proliferation after 6 days of culture of MCF-7 cells in the presence of 0.1% ethanol. This enhanced proliferation is confirmed by the use of clonogenic assays which show a 1.5-fold increase in clonal growth in the presence of 0.1% ethanol. No statistically significant changes were observed in the presence of higher ethanol concentration (0.3%). After a 6-day exposure to 0.1% ethanol, RT-PCR analyses reveal a 1.7-fold increase in ERα mRNA that was not significant, whereas Western blot analyses show a significant 3.3-fold increase in ERα content. At the same stage, RT-PCR studies demonstrate a 2.4-fold increase in aromatase mRNA level which is confirmed at the protein level by Western blots performed after immuno-precipitation of the enzyme. Taken together, these results are in agreement with the involvement of ER signalling in ethanol-induced stimulation of breast cancer cell proliferation and could help to understand why alcohol consumption is associated with breast cancer risk.

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Introduction

Breast cancer is the most prevalent type of cancer among women in the world (30% of all female cancers). One hundred and sixty thousand new breast cancers are diagnosed each year in Europe (1). About 70% of human breast cancers are related to sex hormone exposure and 60% of all patients have hormone-dependent breast cancer. These tumours express estrogen receptors (ER) and require estrogens for tumour growth (2-4). These hormones exert their proliferative effect through the binding to their nuclear receptors which are members of the steroid/thyroid nuclear receptors family, known to have a key role in cell proliferation and differentiation (5). The presence or the lack of ER expression is an important determinant for both prognosis and choice of treatment. A poor prognosis and resistance to endocrine therapy are associated with ER-negative disease (6). The proportion of patients with hormone sensitive tumours is higher among post-menopausal (70%) than in pre-menopausal patients (60%) (7,8). In the former population, breast tumours display a high level of estrogens despite the low plasma level. Previous studies have demonstrated that the expression of aromatase, the estrogen synthesis rate-limiting enzyme, is increased in breast tumours (9). Aromatase (CYP 19) is a cytochrome P_{450}, catalysing three consecutive reactions converting C19 androgens to aromatic C18 estrogenic steroids. It converts androstenedione and testosterone respectively to estrone and estradiol (10).

Epidemiological data have shown that chronic abuse of alcohol, even with a moderate dosage, increased significantly the incidence of breast cancer (11-14). This increased risk is associated with a higher frequency of ER-positive tumours. In contrast, no correlation or even an inverse correlation has been found for patients exhibiting ER-negative tumours. But so far, the molecular mechanism of alcohol-induced carcino-genesis is not yet understood.

It has been shown that ethanol could enhance cell proliferation and increase the ERα content of ER-positive breast cancer cells (15-17). Alcohol can also stimulate ER signalling in human breast cancer cell lines (18). It has been reported that alcohol consumption increased the level of estrogens and DHEAs both in pre-menopausal and post-

menopausal women (19-21). Besides, it is well known that chronic alcoholic men display a higher estrogen plasmatic level leading to severed side effect including azoospermia (22,23). Hence, we proposed that aromatase expression could be increased in cases of alcohol intake, providing a source of estrogens which could stimulate the proliferation of malignant clones derived from breast epithelial cells. The aim of our study was to test this hypothesis and to better understand the role played by ethanol in breast cancer.

Materials and methods

Cell culture. MCF-7 human breast cancer cell line was kindly provided by Professor Didier Picard (University of Geneva, Switzerland). Cells were grown at 37˚C under 5% CO_2 in phenol red-free DMEM, supplemented with 5% foetal calf serum, 2 mM L-glutamine and 100 nM E_2.

For soft agar assays, cells were plated in 24-well flat bottomed plates using a two-layer soft agar system with a total of 10^3 cells/plate in a volume of 500 µl/well as previously described (24).

For cell proliferation assays, cells were seeded in 6-well plates at a density of $2x10^4$ cells/well in 2 ml of medium lacking ethanol. After 24 h of cell attachment, the medium was replaced and supplemented with ethanol at different concentrations (0.0, 0.1 or 0.3%). Cells in triplicate dishes per treatment group were exposed to ethanol for periods up to 6 days. Fresh ethanol-containing medium was added to cells daily. At the end of the treatment, cells were washed with PBS, trypsinized and counted with the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega).

Semi-quantitative RT-PCR. The analysis of P_{450}-aromatase and ERα mRNA expression was performed by RT-PCR. At the end of the treatment, total RNA was extracted from cells using TRIzol reagent (Invitrogen, Basel, Switzerland) as suggested by the manufacturer. cDNA was synthetized in a 20-µl volume containing 2 µg RNA, 1.5 µg random hexamer primer, 1X Invitrogen buffer, 2 mM $MgCl_2$, 7.5 mM DTT, 0.25 mM of each dNTP, 20 U RNase inhibitor, and 200 U of MMLV Reverse transcriptase (Invitrogen, Cergy Pontoise, France). The mixture was incubated for 60 min at 37˚C and subsequently for 5 min at 94˚C in order to stop the reaction.

The cDNAs obtained were further amplified by PCR. The sequences of primer were 5'-CTACATCATCTCGGTTC CGC-3' and 5'-CACCACGTTCTTGCACTTCATG-3' for ERα (25), 5'-CTGGAAGAATGTATGGACTT-3' and 5'-GA TCATTTCCAGCATGTTTT-3' for P_{450}-aromatase (26) and 5'-TACATGGGTGGGGTGTTGAA-3' and 5'-AAGAGAGG CATCCTCACCCT-3' for ß-actin (27). PCR was carried out in a thermal cycler in a final volume of 30 µl containing 2 µl cDNA, 1X Invitrogen Taq buffer, 1.5 mM $MgCl_2$, 125 µM of each dNTP, 0.33 µM of each primer, and 1.5 U Taq polymerase (Invitrogen). Negative controls were always included. Amplification was carried out under the following conditions: 94˚C for 5 min, 25-35 1-min cycles at 94˚C, 1 min at 54˚C, and 1 min at 72˚C, followed by a 10-min extension step at 72˚C. A total of 30 µl of the amplified PCR product was mixed with 6 µl of loading buffer and submitted to electrophoresis in a 2% agarose gel at 90 V for 60 min at room temperature. The gel was stained with ethidium bromide, viewed and photographed on an UV-transilluminator (GelDoc 2000, Bio-Rad Laboratories). A software package (Quantity One v.4.3.1, Bio-Rad Laboratories) was used to analyse the PCR products.

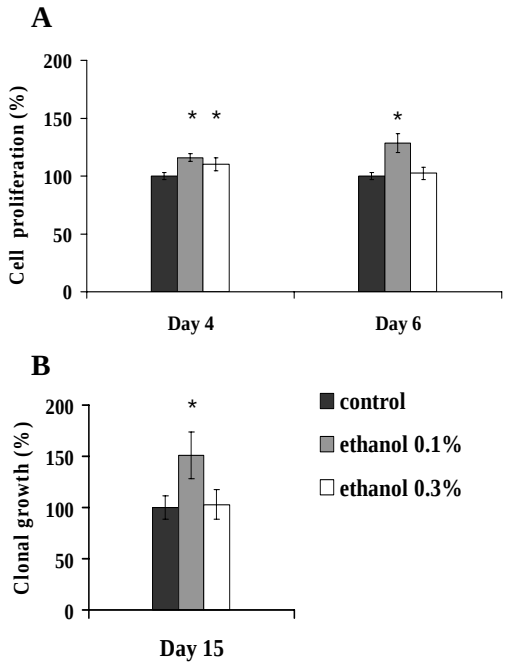

Figure 1. (A), Effect of ethanol concentration on proliferation of MCF-7 cells. At the indicated time of treatment, cell numbers were evaluated using the CellTiter-Glo™ Luminescent Cell Viability Assay. Results are given as mean ± standard error of 6 different counts. (B), Effect of ethanol concentration on clonal growth of MCF-7 cells. Results are given as mean ± standard error of 3 different experiments. *Significantly different from untreated control (Student's t-test, $p<0.05$).

Electrophoresis and Western blotting. MCF-7 cells were plated in 75 cm^2 flasks at $2x10^5$ cells/flask and exposed to ethanol. At the end of the treatment, they were washed twice with cold PBS and collected in extracting buffer containing 50 mM Tris, 10 mM DTT, pH 6.8, 2% SDS and 10% glycerol. After heating (100˚C, 5 min), an aliquot was used for protein concentration determination using the RC-DC reagent (Bio-Rad Laboratories). For each sample, 50 µg of total proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were subjected to Western blot analysis as previously reported (28). ERα was detected using the monoclonal antibody F-10 (SC-8002, Santa Cruz) and for tubulin, a mouse monoclonal antibody against acetylated tubulin was used (clone 6-11B-1, Sigma). Aromatase was detected using a mouse monoclonal antibody to human cytochrome P_{450}-aromatase (clone H4, Serotec).

Immunoprecipitation. Immunoprecipitation experiments were carried out to study aromatase. At the end of each treatment, MCF-7 cells were lysed on ice in 500 µl of a buffer containing 1% Triton X-100, 150 mM NaCl, 10% glycerol, 0.1% SDS, 50 mM HEPES, 5 mM NaF, 2 mM EDTA, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor, 100 µM benzamidine, pH 7.4. Samples were

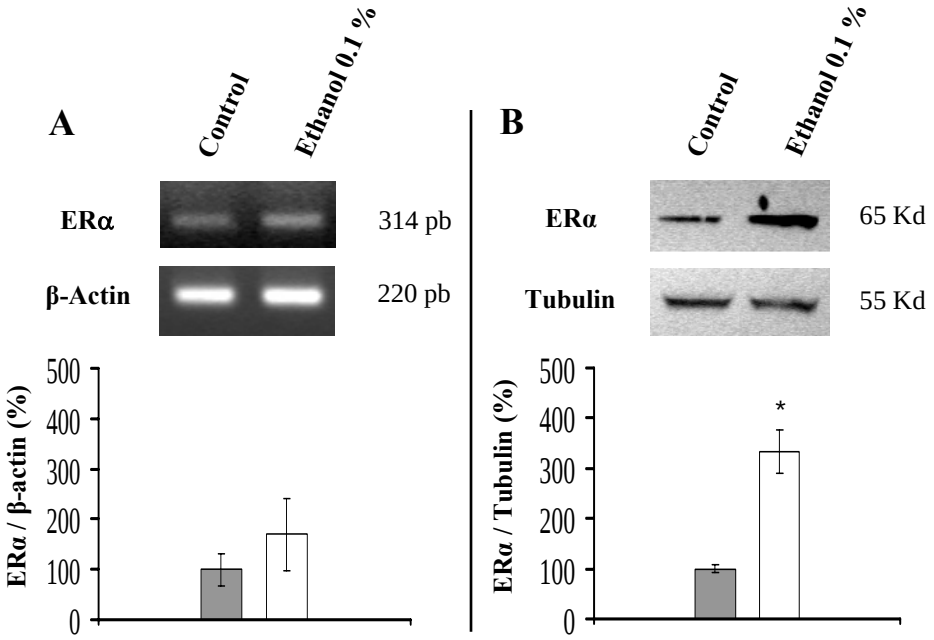

Figure 2. Expression of ERα in control versus ethanol-treated MCF-7 cells (0.1% for 6 days). (A), mRNA analysis. At the top of the figure is shown the result of a representative RT-PCR experiment. At the bottom is shown the result of the semi-quantitative analysis performed on 6 different experiments. The 314 bp amplified ERα product was quantified by densitometry and expressed relative to the control gene, ß-actin. (B), Protein analysis. At the top is shown the result of a typical Western blot whereas at the bottom is shown the result of the semi-quantitative analysis performed on 3 different experiments. ERα was expressed relative to the control protein, tubulin. *Significantly different from control (Student's t-test, $p<0.05$).

centrifuged (10,000 g, 10 min at 4˚C) and the supernatants were removed and spun again for 10 min before total protein content determination using the RC-DC reagent (Bio-Rad Laboratories).

Each sample was diluted to a total protein concentration of 1 µg/ml in TBST buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.4). In order to preclear the samples, they were incubated with 20 µl of a 50% slurry of protein A-sepharose beads (Sigma) in TBST and rocked for 10 min at 4˚C. Samples were then spun (10,000 g, 10 min, 4˚C). Supernatants were removed and 500 µl of each sample was incubated rocking overnight with 5 µl of rabbit anti-human placental P_{450}-aromatase serum (R-11-4, Hauptman Woodward Institute, Buffalo, USA). Immunocomplexes were precipitated with a 2-h incubation with 10 µl of TBST-washed protein A-sepharose beads that were then spun at 4˚C for 15 sec at 10,000 g. After washing in TBST, the pellet was resuspended in 50 µl of Laemmli's sample buffer and submitted to electrophoresis and Western blotting as described above.

Statistical analysis. The results are expressed as mean ± standard error of several experiments as indicated in the text. Data were compared by means of a Student's t-test; significance level at $p<0.05$.

Results

Ethanol enhances cell proliferation and clonal growth of MCF-7 cells. First, we evaluated the effects of ethanol on MCF-7 cell proliferation. Two sets of cells were incubated in the presence of 0.1 or 0.3% ethanol. Relative cell proliferation was measured after 4 and 6 days of treatment and compared to untreated control cells. When MCF-7 cells were grown in the presence of 0.1% ethanol, a significant increase in cell proliferation (11.5%) was observed following 4 days of culture and it peaked at 28% after 6 days of treatment ($p<0.05$) (Fig. 1A). In contrast, in the presence of 0.3% ethanol, after a transient significant increase (11%) at day 4, no significant change was observed at day 6.

Then, we measured the effects of ethanol on MCF-7 cell proliferation in conditions of anchorage-independent growth using soft agar clonogenic assays. Ethanol (0.1%) induced a 50% increase in MCF-7 clonal growth (Fig. 1B). In correlation with the results of cell proliferation analysis in liquid culture, no significant change was observed in the presence of 0.3% ethanol.

Increase in ERα *and aromatase expression in the presence of ethanol*. Then, we investigated the molecular mechanisms underlying this enhanced cell proliferation. It is well described that breast epithelial cell proliferation is mediated by the binding of estrogens to their nuclear receptor ERα (29-31). So, we tested if ethanol treatment could modify the level of ERα in our MCF-7 cell line.

MCF-7 cells were incubated in ethanol containing medium and after 6 days of treatment, the cells were harvested for mRNA and protein analysis. Semi-quantitative RT-PCR analyses of ERα mRNA content revealed a general upward trend (1.7-fold increase) which was not significant ($p>0.05$) whereas Western blot studies showed a 3.3-fold increase in the ERα protein level as compared to controls ($p<0.05$) (Fig. 2).

In order to determine whether ethanol treatment could stimulate aromatase expression, semi-quantitative RT-PCR studies were performed. We observed a 2.4-fold increase in aromatase mRNA level in MCF-7 cells that were grown during

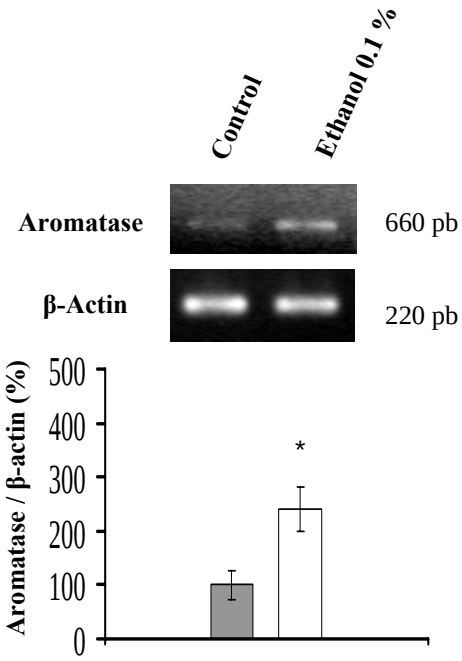

Figure 3. Expression of P_{450}-aromatase mRNA in control versus ethanol-treated MCF-7 cells (0.1% for 6 days). At the top of the figure is shown the result of a representative RT-PCR experiment. At the bottom is shown the result of the semi-quantitative analysis performed on 4 different experiments. The 660 bp amplified aromatase product was quantified by densitometry and expressed relative to the control gene, ß-actin. *Significantly different from control (Student's t-test, $p<0.05$).

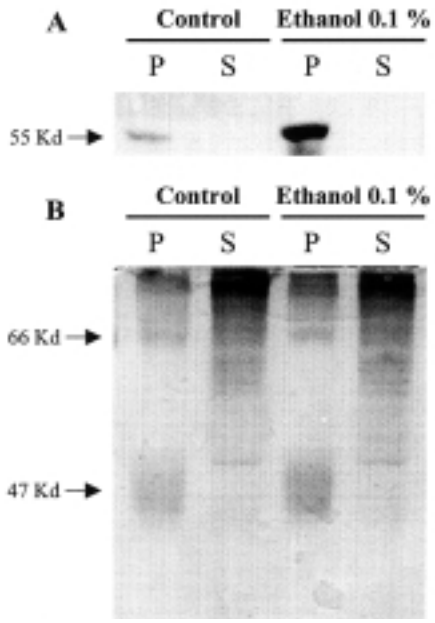

Figure 4. Expression of P_{450}-aromatase in control versus ethanol-treated MCF-7 cells (0.1% for 6 days). (A), Western blot showing aromatase in immunoprecipitates. A signal at 55 kDa is detected by the antibody only in pellets (P) but not in supernatants (S), suggesting that aromatase was totally immunoprecipitated. The intensity of this signal is higher in ethanol-treated cells. (B), Coomassie blue staining of the corresponding gel after the transfer. This staining shows that the total protein content is similar in ethanol-treated versus control cells.

6 days in the presence of 0.1% ethanol ($p<0.05$) (Fig. 3). To confirm this result at the protein level, several Western blot analyses were performed. We always failed to detect aromatase in total homogenates of MCF-7 cells, even when 100 µg of total proteins had been loaded at the top of the polyacrylamide gel. Aromatase was detected only after immunoprecipitation. The result of a typical experiment is shown in Fig. 4. The intensity of the band revealed by the antibody at 55 kDa in the sample of ethanol-treated MCF-7 cells was higher than those in the control (Fig. 4A). As shown by the Coomassie blue staining of the gel after transfer, the two supernatants were identical regarding their total protein content (Fig. 4B).

Discussion

It is well documented that ethanol is associated to an increased risk factor for breast carcinogenesis although the underlying mechanisms are not clearly understood (11-14). It has been reported that *in vitro*, the culture of ER-positive breast cancer cells in ethanol containing medium was associated with an increase in the proliferation rate whereas no effect was obtained on ER-negative breast cancer cells (16). Interestingly, similar results were observed on hepatocytes which are very sensitive to sex steroid hormones. In male rats, chronic intake of ethanol increased 3-fold the number of ERα-positive hepatocytes and was associated with an elevation of hepatocyte proliferation ability (32,33). In human, active alcohol intake is required to maintain high level of ER-positive hepatocytes. Moreover, alcohol intake has been associated to an increased level of circulating estrogens (34). Hence, we have postulated that the stimulation of proliferation observed in ER-positive breast cancer cells following ethanol exposure could be the result of an elevation of local estrogen level through an effect on P_{450}-aromatase expression. The results of our studies confirm the stimulation of the proliferation rate and the increase in ERα content previously reported. Besides, they demonstrate for the first time an increase in aromatase mRNA level following ethanol treatment.

First, it was necessary to re-evaluate on our cell line the effect of ethanol on MCF-7 proliferation reported previously. When MCF-7 cells were grown in DMEM containing 5% FCS, the most significant increase in proliferation was observed after 6 days of treatment with 0.1% ethanol. A shorter exposure to ethanol (4 days) as well as treatment with a higher dose of ethanol (0.3%) appeared less efficient. In the previous studies, various results were obtained. An increase in [^{3}H]-thymidine incorporation was measured after 5 h of exposure to ethanol in DMEM containing 10% FCS and the most efficient concentration was 0.01% (15). An increase in cell number was also reported after 7 days of exposure of MCF-7 to ethanol at concentrations between 10 and 100 mM (i.e. between 0.025 and 0.5%) in medium containing 5% FCS (16). The increase reached 54 and 87% in the presence of respectively 20 and 100 mM. However, the authors did not observe any change after 2 days of treatment. Lastly, an increase in [^{3}H]-thymidine incorporation and in cell number was measured after 24 h of treatment with ethanol at concentrations ranging from 0.1 to 10% in serum-free medium (17). In that study, the highest effect (60% increase) was observed in the presence of 0.3% ethanol. In our study, the concentration of 0.3% of ethanol was not more efficient than 0.1% in stimulation of

cell proliferation, even in clonogenic assays. This is probably not the result of a toxic effect because using trypan blue, we did not observe a higher number of cell membrane permeabilization in cultures performed in the presence of 0.3% ethanol (data not shown). The lack of toxicity of this concentration was also reported previously using the MTT method (35). It should be noted that, for instance, women consuming daily 0.75 g alcohol per kg of body weight have blood ethanol concentrations of 20-24 mM i.e. approximately 0.1% (16). So, despite the differences in the most efficient dose and the time at which the proliferative effect is observed, our results are in agreement with those reported in other studies and confirm the proliferative effect of ethanol on ER-positive breast cancer cells.

Besides, using clonogenic assays, we observed that MCF-7 displayed a 1.5-fold increase in anchorage-independent growth after treatment with 0.1% ethanol. This result could be an important indicator for tumour progression and reinforce the fact that ethanol is also able to stimulate cell proliferation.

Second, we completed the studies on the effect of ethanol on ERα expression that were only performed by immunodetection of the protein. Our Western blotting analyses showed that the level of ERα was 3.3-fold higher in MCF-7 cells that were grown during 6 days in the presence of 0.1% ethanol. A 1.6-fold increase in ERα was previously reported to occur as early as at 24 h of incubation in medium containing 20 mM ethanol (18). Another study failed to detect any change at such an early time of treatment although significant changes were found later: a 2.1-fold level of ERα was measured after 7 days of culture in the presence of 20 mM ethanol (16).

Although the increase in ERα at the protein level was clearly observed and was statistically significant in our experiments, a 1.7-fold increase in the level of ERα mRNA was observed but it was not significant. So, it was difficult to conclude that there is a stimulation of the transcription following ethanol treatment. The subcellular trafficking process may influence receptor stabilisation and it is well known that the binding of estrogens induces ER phosphorylation, increases its nuclear translocation and consequently its stabilisation (36-39). This explanation could reinforce the idea that aromatase, the estrogen synthesis enzyme, could be modified in MCF-7 cells subjected to ethanol treatment.

Indeed, alcohol consumption has been associated to an increase in plasma estrogens level in post-menopausal women (34,40). A clinical study of post-menopausal women under estrogen replacement therapy showed that estrogens level peakes at 300% from the baseline value after alcohol absorption (0.7 g/kg) (41). Moreover, some direct or indirect indications of aromatase involvement in ethanol-induced increased level of estrogens have been described for various peripheral tissue (42). For instance, in male rat, an increase in hepatic aromatization has been observed after exposure to an alcohol enriched diet (36% calories) (33). In male rat pup, foetal exposure to ethanol displays a significant increase in aromatase activity (43). Here, we show evidence for the first time that P_{450}-aromatase is involved in ethanol signalling pathway in breast cancer cells, since a 6-day exposure to ethanol containing medium induced a 2.4-fold increase of the mRNA level of this enzyme. Moreover, we found a 10.1-fold increase in the amount of the protein in ethanol-treated MCF-7 cells. This result was obtained using immunoprecipitation experiments. This suggests that the amount of aromatase is initially very low in MCF-7 cells and this is consistent with the fact that 35 cycles were required to detect the endogenous mRNA by RT-PCR.

The involvement of aromatase in breast cancer is well established. Aromatase overexpression in some breast carcinoma has been detected in epithelial tissue, in adipose tissue and in stromal cells (44-47). It is also known that transgenic mice overexpressing P_{450}-aromatase display several premalignant changes such as hyperplasia and dysplasia in the epithelial cells of the mammary gland (7). As a consequence, through the increase in aromatase expression in breast tumour, ethanol could play a key role in malignant breast cancer cell proliferation.

How could the ethanol-induced increase be explained in aromatase expression? It has been reported that I.3 and II are the two major promoters directing aromatase expression in breast cancer cells (9). Both of these promoters are sensitive to cAMP (48,49). Since ethanol treatment of MCF-7 cells has been shown to increase cAMP amount (16), this might be the reason for the enhanced expression of P_{450}aromatase.

Furthermore, recent studies show that in rat, ethanol is metabolised by the breast microsomes into a mutagen (acetaldehyde) (50). If a similar process occurs in human breast normal cells, this mutagen could be the starting point of carcinogenesis. Indeed P53 is frequently mutated (51,52) in breast carcinoma suggesting an effective involvement of carcinogen but, so far, to our knowledge, no specific study has been carried on alcoholic women. In this model, ethanol would both induce a mutagenic process and increase the proliferation of breast cancer cells by boosting the ER signalling pathway.

In summary, this study supports data suggesting that ethanol is an increased risk factor for breast cancer, showing the impact of ethanol on estrogen signalling pathway and evidencing the involvement of P_{450}-aromatase. These data should be considered for women under estrogen replacement therapy for menopause or, more generally, for women with an increased risk of developing cancer. Moreover, the pharmacological tests used in order to evaluate anticancer drugs should take into account the present data when ethanol is used to solubilize drugs such as nuclear receptor ligands.

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