Hydroxylated estrogen metabolites influence the proliferation of cultured human monocytes: possible role in synovial tissue hyperplasia

S. Capellino[1, 2], P. Montagna[1], B. Villaggio[1], S. Soldano[1], R.H. Straub[2], M. Cutolo[1]

[1] Research Laboratory, Academic Clinical Unit of Rheumatology, Postgraduate School of Rheumatology, University of Genova, Genova, Italy; [2]Laboratory of Experimental Rheumatology and Neuroendocrino-Immunology, Department of Internal Medicine I, University Hospital Regensburg, Regensburg, Germany.

Abstract

Introduction

17β-estradiol, estrone, and several of their hydroxylated metabolites, have been found to be significantly increased in synovial fluid of rheumatoid arthritis (RA) patients. In this study, we investigated whether the estrogen metabolites are able to exert direct effects on monocyte cell proliferation, which is important in RA synovial tissue activation and growth.

Methods

Human monocytes (THP-1) were treated with the following estrogen metabolites at different concentrations (from 10^{-8}M, 10^{-9}M, 10^{-10}M to 10^{-11}M) for 24, 48 and 72 hours: 16-hydroxyestrone (16OH-E1), 16-hydroxyestradiol (16OH-E2), 4-hydroxyestrone (4OH-E1), 4-hydroxyestradiol (4OH-E2), 2-hydroxyestrone (2OH-E1) and 2-hydroxyestradiol (2OH-E2). Monocytes were activated with interferon-γ (INF-γ). Cell cultures were also performed in presence of tamoxifen (10^{-7}M) to evaluate whether the estrogen metabolites act through the estrogen receptors (ER). Cell growth was detected by MTT test and cell viability through the LDH release assay.

Results

4OH-E1 and 2OH-E1 significantly increased cell growth at low concentration (10^{-10}M), whereas they significantly reduced cell proliferation at high concentrations (10^{-9}M). 16OH-E2 and 4OH-E2 induced opposite effects: cell proliferation at high concentration and antiproliferative action at low doses. On the contrary, 16OH-E1 and 2OH-E2 were found to be estrogen metabolites that induced cell proliferative effects for most of the tested doses. Tamoxifen caused the loss of effects on cell proliferation for almost all the metabolites.

Conclusions

This study first demonstrates that different downstream estrogen metabolites interfere with monocyte proliferation and generally might modulate the immune response. Therefore, since estrogen metabolite/ratios are altered in the synovial fluid of RA patients, they might play important roles at least in RA synovial tissue hyperplasia.

Key words

Hydroxy-estrogen metabolites, estrogens, monocytes, synovitis, synovial tissue hyperplasia, rheumatoid arthritis.

Silvia Capellino, PhD
Paola Montagna, PhD
Barbara Villaggio, PhD
Stefano Soldano
Rainer H. Straub, MD
Maurizio Cutolo, MD

This work is to be attributed to the Research Laboratory, Academic Clinical Unit of Rheumatology, Postgraduate School of Rheumatology, University of Genova, Genova, Italy.

Please address correspondence and reprint requests to:
Dr. Maurizio Cutolo, Research Laboratory, Academic Clinical Unit of Rheumatology, Postgraduate School of Rheumatology, University of Genova, Viale Benedetto XV 6 16132 Genova, Italy.
E-mail: mcutolo@unige.it

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List of abbreviations:
RA: rheumatoid arthritis
SLE: systemic lupus erythematosus
INF-γ: interferon-γ
MTT: bromure of 3(4, 5-dimethylthiazol-2l)-2, 5-diphenyl-tetrazolium
LDH: lactate dehydrogenase
FBS: fetal bovine serum
E2: 17β-estradiol
h: hours
cnt: control

Competing interests: none declared.

Introduction

An increased susceptibility of women for most autoimmune diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), is well recognized (1, 2). This clinical evidence supports an important role that sex hormones play in the regulation of the immune response and cell proliferation (3). Indeed, many cells involved in the immune system, such as monocytes or synovial macrophages, have been found to possess functional sex hormone receptors and several *in vitro* studies clearly demonstrated that estrogens play a key role in the pathophysiology of RA, as general enhancers of the immune response (4-6). In addition, an increased estrogen/androgen ratio has been found in the synovial fluid not only of RA women, but also of men affected by RA, suggesting a correlation between local estrogen levels and the disease, not related to gender (6-9).

Furthermore, in recent years there has been a growing interest not only in 17β-estradiol and estrone, as the two major estrogens, but also in a group of hydroxylated estrogen metabolites as important modulators of cell growth (7). In the peripheral tissue, estrogens are converted into active hydroxylated compounds and some of them have been found to be increased in RA synovial fluid (8). In particular, estrone is converted into 2-hydroxyestrone (2OH-E1), 4-hydroxyestrone (4OH-E1), and 16-hydroxyestrone (16OH-E1), while 17β-estradiol is converted into 2-hydroxyestradiol (2OH-E2), 4-hydroxyestradiol (4OH-E2) and 16-hydroxyestradiol (16OH-E2) (Fig 1).

Interestingly, breast cancer research revealed a mitogenic tumor growth-stimulating role of 16-hydroxylated estrogens, supporting the potent proliferative activity of these metabolites (10). 16-hydroxyestrone covalently binds to the estrogen receptor and thereby induce nuclear translocation of the hormone-receptor complex and subsequently growth of breast cancer cells *in vitro* (11). Also 16-hydroxyestradiol has proliferative effects on human osteoblasts, whereas it has no effects on cell differentiation (12). On the contrary, 2-hydroxyestrone seems to exert anti-proliferative effects, at least on breast cancer cells (13). The effects of 4-hydroxylated estrogens are still unclear, although there are studies highlighting a possible role of these hormones (14, 15).

Recently, it has been demonstrated that patients affected by systemic immune-inflammatory diseases such as RA and SLE, present an altered ratio for several of these metabolites (8, 16). These results further suggest a role for estrogen metabolites in the systemic inflammatory diseases, and their possible important modulatory role at least in the pathophysiology of RA (synovitis) should be considered.

Therefore, the aim of this study was to investigate whether hydroxylated estrogen metabolites exert direct effects on proliferation/activation of human monocytes, which is recognized as an important mechanism of synovial tissue inflammation and hyperplasia at least in RA (16).

Methods

Hormonal cell treatment and monocyte proliferation

THP-1 human monocytic cells are equipped with functional estrogen receptors and they acquire phenotypic and functional macrophage-like features after treatment with several cytokines or pharmacological agents, such as in primary culture of human monocytes (4). Therefore, we considered this cell line reliable for our *in vitro* studies (4).

Monocytic cells were cultured in 24 well microplates (50,000 cells/ml) in RPMI medium with 5% FBS, 1% penicillin/streptomycin, and 5 nM ascorbic acid (all products from Sigma Aldrich). 16OH-E1, 16OH-E2, 4OH-E1, 4OH-E2, 2OH-E1 or 2OH-E2 (all hormones from Steraloids Inc., Rhode Island, USA.) were tested at different concentrations (10^{-8} M, 10^{-9} M, 10^{-10} M and 10^{-11} M) for 24, 48 or 72 hours. These concentrations are similar or lower to the concentration of 17-estradiol detectable in the synovial fluid of RA patients (8). As controls, further THP-1 cells were treated under the same conditions but without hormones. At the end of the different culture times, cells were activated with interferon-γ (INF-γ) (500

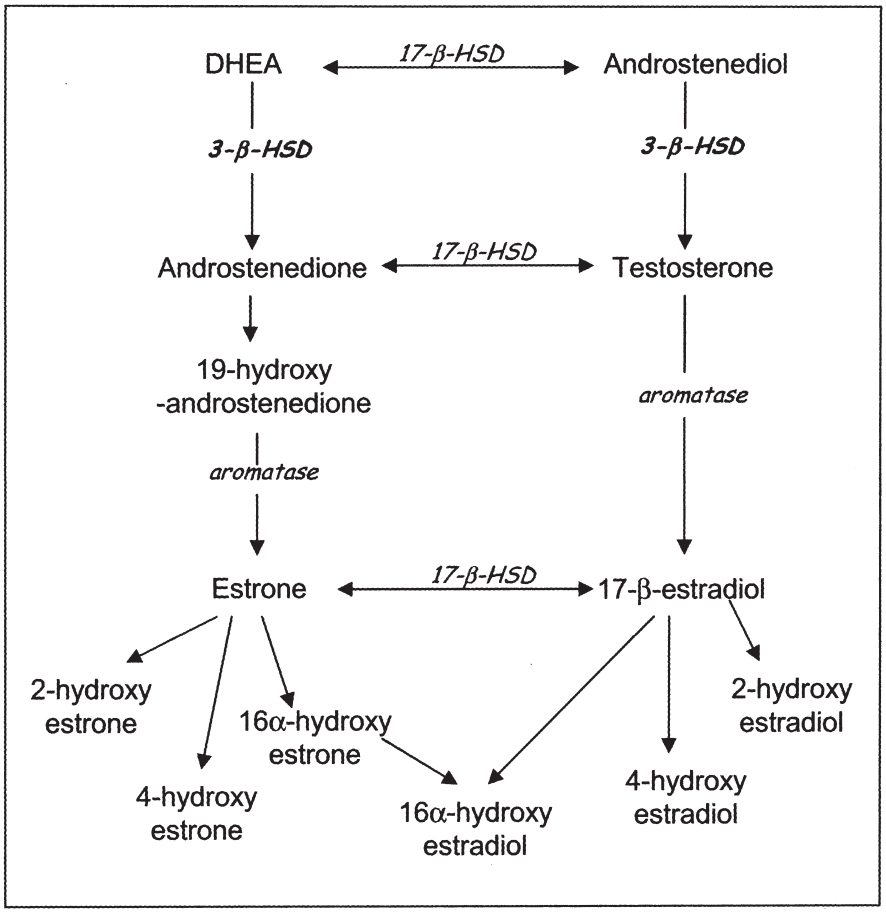

Fig. 1. Biosynthesis of steroid hormones.
Precursor hormones (DHEA, androstenediol) are converted to androgens (androstenedione, testosterone) and estrogens (estrone, 17β-estradiol). Estrogens are then converted to downstream hydroxylated metabolites. Enzymes: 3-β-HSD: 3β-hydroxysteroid dehydrogenase; 17-β-HSD: 17β-hydroxysteroid dehydrogenase.

U/ml) (Sigma Aldrich, Milan, Italy) for further 24 hours. Cells treated for more than 48 hours were divided (1:2) after 48 hours, in order to avoid overgrowth. At the end of the culture, samples were harvested. 300 µl of each sample were used for the MTT proliferation assay (see below), then the samples were centrifuged (1600 rpm, 10 minutes at 4°C). Supernatant culture medium was collected and two aliquots of each sample were stored at -20°C until LDH assay (see below).

Use of ascorbic acid as antioxidant

To prevent oxidation and inactivation of hydroxylated estrogen metabolites, an antioxidant compound was required in the cell cultures. We decided to use ascorbic acid as antioxidant, and we evaluated the optimum dose for further experiments in order to prevent oxidation without altering the readout-parameters (cell growth and viability). Human monocytic cells (THP-1). were cultured in 24 well microplates and treated with different concentrations of ascorbic acid (Sigma Aldrich, Milan, Italy) (0.10, 0.05, 0.025 and 0.005 mM) for 6, 24, 48 or 72 hours. Cells treated for the longest incubation time (72 hours of culture) were divided in further wells (1:2) after 48 hours of culture, to avoid overgrowth and consequently alterations of cell growth and viability. At the end of the culture, cells were harvested and counted with Trypan Blue, to judge viability. As controls, THP-1 were cultured under the same conditions but without ascorbic acid.

MTT test

The MTT test is a colorimetric assay based on the evaluation of the succinate dehydrogenase activity. This mitochondrial enzyme transforms bromure of 3 (4, 5-dimethylthiazol-2l)-2, 5-diphenyltetrazolium (MTT) into formazan. As the number of mitochondria does not significantly differ in the cells, the activity of this mitochondrial enzyme can be correlated with the number of viable and proliferating cells. MTT test was performed using a 96 well microplate and the Cell Titer Aqueous One solution (Promega, Milan, Italy). THP-1 cells cultured in medium without treatment but with ascorbic acid were seeded in the microplate at different concentrations and used as standard curve ($5x10^5$, $2.5x10^5$, $1x10^5$, $5x10^4$, $1x10^4$ and $5x10^3$ cells/well, each concentration in triplicate). Samples with the different metabolite treatments were seeded in the others wells, 100 µl each well, in triplicate, and diluted (1:2) with normal RPMI + ascorbic acid. In each well 20 µl of MTT One Solution was added. After 3 hours of incubation at 37°C in a humidified atmosphere at 5% CO_2, absorbance at 490/620 nm was measured. From the mean O.D. of each sample, the respective cell number was calculated using the above-mentioned standard curve. Cell culture and MTT test were performed three times, and the final results for each treatment corresponded to the mean of the three experiments.

LDH test

As confirmation that the tested metabolites were not toxic even during prolonged incubation and high concentration, LDH activity test was performed on 72 hours culture samples treated with the different hydroxyestrogens at the concentration of 10^{-8} M. The LDH release in the culture medium is a valid indicator of cell membrane damage due to toxicity (17). To evaluate the LDH release, we used a solution of L-lactic acid (sodium salt) at $5.4x10^{-2}$ M, β-nicotinamide adenin dinucleotide (β-NAD) at $1.3x10^{-3}$ M, piodonitrotetrazolium violet at $6.6x10^{-4}$ M and phenazine methosulfate (PME) at 2.8×10^{-4} M (all reagents from Sigma Aldrich, Milan, Italy). 100 µl of this solution and 100 µl of supernatant medium (all samples in triplicate) were added into a 96 well microplate. As control, supernatant medium of THP-1 cells cultured without hormones was used, while as

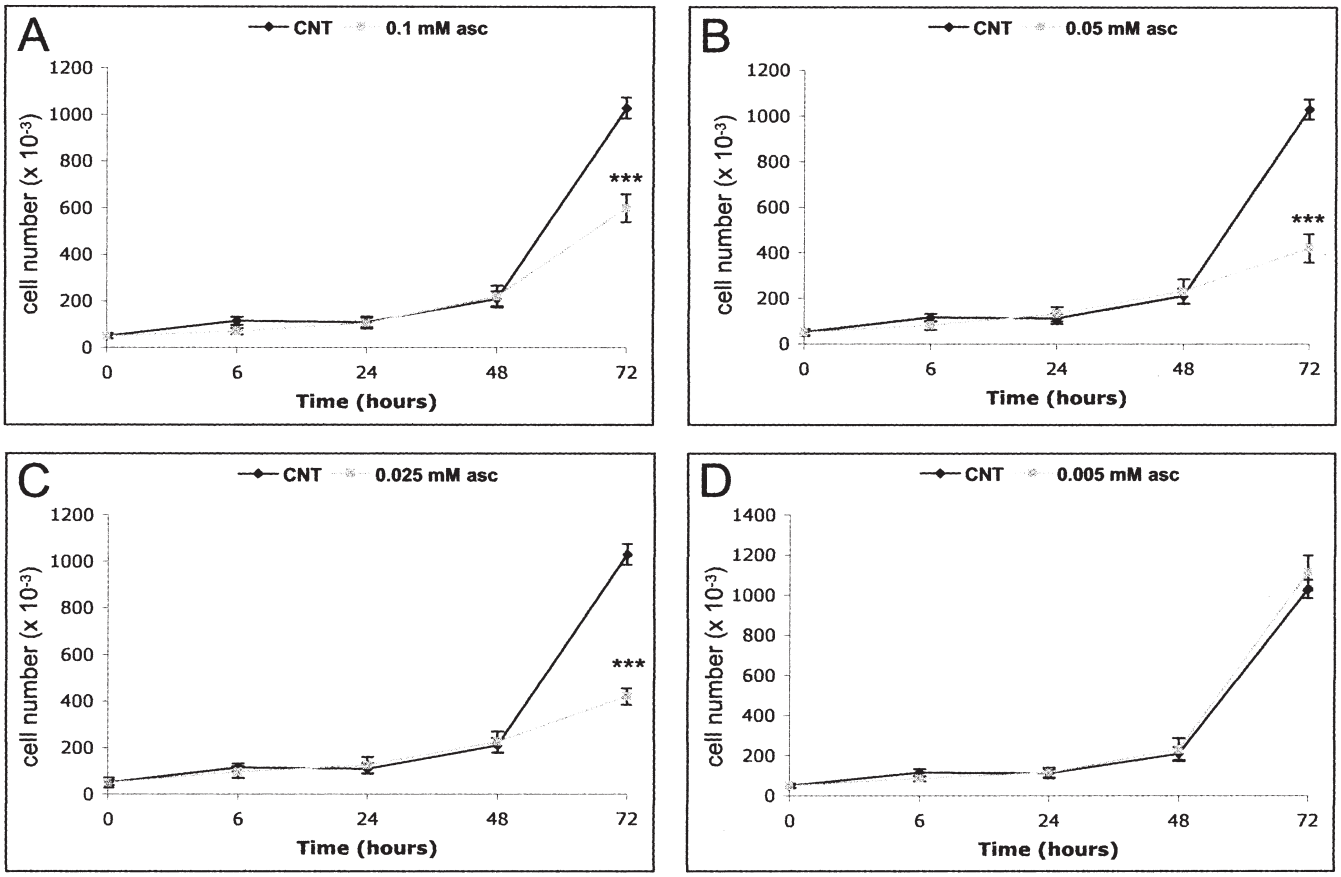

Fig. 2. Effects of ascorbic acid on cell growth.
Cell growth after treatment with ascorbic acid 0.1 mM (A), 0.05 mM (B), 0.025 mM (C) and 0.005 mM (D) for 6, 24, 48 and 72 hours. CNT: untreated control THP-1 cells; asc: THP-1 cells treated with ascorbic acid. Data are given as means±SEM. ***p<0.001.

blank, fresh RPMI medium+ascorbic acid was used. The plates were incubated at 37°C in a humidified atmosphere at 5% CO_2. Absorbance at 490/620 nm was measured every 20 minutes, and the mean OD of each sample, after normalization to cell number obtained by MTT test, was evaluated. Cell culture and LDH test were performed three times, and the final results represent the mean of the three experiments.

THP-1 cell culture with estrogen metabolites and tamoxifen

The monocytic cells were cultured as already described, with 16OH-E1, 16OH-E2, 4OH-E1, 4OH-E2, 2OH-E1 or 2OH-E2 at different concentrations (10^{-8} M, 10^{-9} M, 10^{-10} M and 10^{-11} M) and in the presence of tamoxifen (10^{-7} M) for 72 hours. As tamoxifen is rapidly degraded, we added fresh tamoxifen in the cell culture every 24 hours. In order to act as controls, further THP-1 cells were treated under the same conditions but without estrogen metabolites. At the end of the culture, cells were activated with interferon-γ (INF-γ) (500 U/ml) for a further 24 hours. Cells treated for more than 48 hours were divided in further wells (1:2) in order to avoid cell overgrowth. At the end of the culture period, cells were harvested and used to perform the MTT test.

Table I. Summary of the observed cell counts (72 hours of culture) on addition of increasing concentrations of estrogen metabolites and the relative values (RVs) compared to the control counts. All values are to be intended x10^6. Each result represents the mean value of three independent experiments. *p<0.05; **p<0.005; ***p<0.001.

	Estrogen metabolite concentrations			
Metabolites control count	10^{-11} M Counts and RVs	10^{-10} M Counts and RVs	10^{-9} M Counts and RVs	10^{-8} M Counts and RVs
20H-E1	1803	2225	980	1949
1648	1.09	1.35***	0.59***	1.18
20H-E2	2172	2307	1932	2682
1648	1.32***	1.40**	1.17	1.63
40H-E1	1568	2403	1312	1198
1648	0.95	1.46***	0,80***	0.73
40H-E2	1799	967	2473	1648
1648	1.09	0.59**	1.50**	1.00
16OH-E1	1970	2349	1876	1593
1648	1.20	1.43**	1.14*	0.97
16OH-E2	1942	1355	2785	2077
1648	1.18	0.82	1.69***	1.26

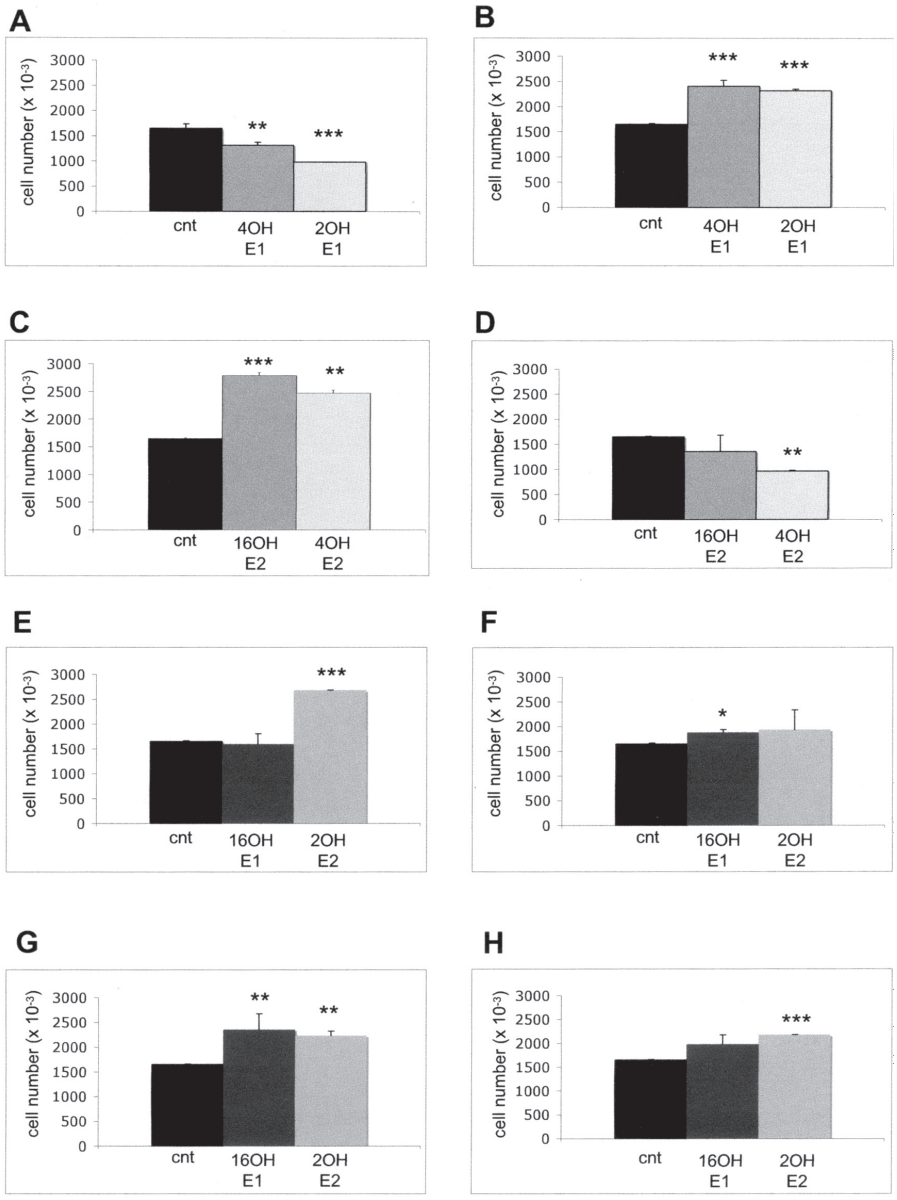

Fig. 3. Cell growth after 72 hours of culture with estrogen metabolites, evaluated by MTT test. Each result represents the mean value of three independent experiments. Control samples (cnt) are cells cultured without estrogen metabolites in the medium. $^{*}p$ <0.05; $^{**}p$<0.005; $^{***}p$<0.001.
3A-B: Cell proliferation after treatment with 4-hydroxyestrone (4OH-E1) and 2-hydroxyestrone (2OH-E1) 10^{-9} M (A) and 10^{-10} M (B).
3C-D: Cell proliferation after treatment with 16-hydroxyestradiol (16OH-E2) and 4-hydroxyestradiol (4OH-E2) 10^{-9} M (C) and 10^{-10} M (D).
3E-H: Cell proliferation after treatment with 16-hydroxyestrone (16OH-E1) and 2-hydroxyestradiol (2OH-E2) 10^{-8} M (E), 10^{-9} M (F), 10^{-10} M (G) and 10^{-11} M (H).

Statistical analysis

The values are expressed as mean ± standard deviation. Statistical analysis was carried out using the parametric *t*-test. A *p*-value equal or less than 0.05 was considered statistically significant.

Results

Effects of antioxidant treatment

Treatment with ascorbic acid 0.10, 0.05 and 0.025 mM induced a significant reduction in cell growth between 48 and 72 hours of culture, when compared with controls without antioxidant treatment (Figs. 2A-2C) (p<0.0001). On the contrary, ascorbic acid at 0.005 mM did not show to alter cell growth and viability even after prolonged incubation for 72 hours (Fig. 2D). Therefore, we decided to use the concentration of 5 nM for the study.

Influence of estrogen metabolites on THP-1 cell proliferation

Cells treated for 24 and 48 hours with the different metabolites showed no significant differences in the proliferation rate when compared to untreated control for all the metabolites and dosages tested (data not shown).

Interestingly, activated monocytic THP-1 cells pre-cultured for 72 hours with 4OH-E1 and 2OH-E1 showed a significant reduction of cell proliferation at the hormonal concentration of 10^{-9} M (-20.4% and -41% *vs.* cnt, p<0.01 and p<0.0001 respectively), and an opposite, proliferative effect at the concentration of 10^{-10} M (+45.7% and +35% *vs.* cnt. p<0.0001 and p<0.0005, respectively) (Figs. 3A-B and Table I). The metabolite concentrations of 10^{-8} M and 10^{-11} M did not induce significant changes in cell proliferation when compared to untreated controls (data shown in Table I).

On the contrary, the hydroxy-estrogens 16OH-E2 and 4OH-E2 showed an opposite action, but again dose-related effects. In fact, after 72 hours of treatment at the concentration of 10^{-9} M, we observed a significant increase of the cell number (+69% and +50% *vs.* cnt. p<0.0005 and p<0.005, respectively), whereas at the lower dose of 10^{-10} M the 16OH-E2 had no significant effect in the cell proliferation. 4OH-E2 induced a significant reduction of cell proliferation (-41.4% *vs.* cnt. p<0.005) (Figs. 3C-D and Table I). Once again, the metabolite concentrations of 10^{-8} M and 10^{-11} M were confirmed not active in the cell proliferation compared to untreated controls (data shown in Table I).

Finally, the 16OH-E1 and 2OH-E2 were the only two metabolites which induced proliferative effect for most of the tested doses. In fact, 16OH-E1 induced proliferation at the concentration of 10^{-9} M and 10^{-10} M. In particular, at the concentration of 10^{-10} M we observed an increase of 42.5% in the cell number compared to the untreated control (p<0.005). 2OH-E2 showed a strong proliferative effect at all the tested concentration: 10^{-8} M +62.7%, 10^{-10} M +40.5% and 10^{-11} M +31.7% *vs.* cnt (p<0.0001, p<0.005 and p<0.0005 respectively) (Figs. 3E-H and Table I).

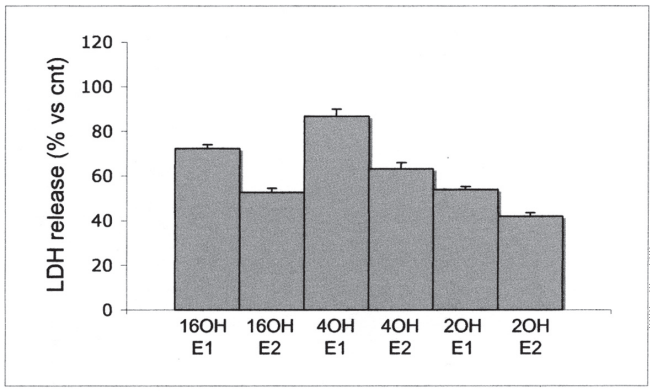

Fig. 4. Effects of 72 hours treatment with estrogen metabolites (10^{-8} M) on cell viability evaluated by LDH release assay (see methods). The results are shown as percentage of LDH release compared to control and normalized to the cell number obtained with the MTT test. Each value represents the mean of three independent experiments.

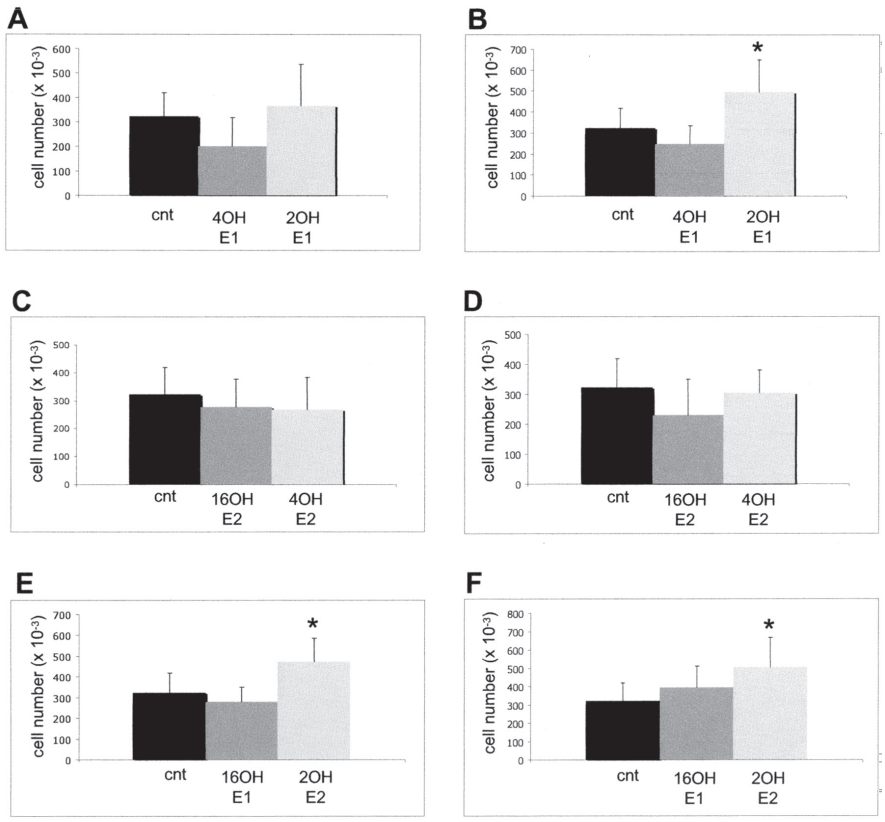

Fig. 5. Effects of tamoxifen 10^{-7} M on cell growth after 72 hours of culture with estrogen metabolites 10^{-9} M (A) and 10^{-10} M (B), evaluated by MTT test. Each result represents the mean value of three independent experiments. Control samples (cnt) are cells cultured with tamoxifen (10^{-7} M) but without estrogen metabolites in the medium. $^{*}p<0.05$.

LDH activity tests, used to check cell viability, confirmed that even the highest metabolite concentrations, employed in this study, did not cause alterations of cell viability. In particular, the release of LDH in the culture medium was always lower, or in some cases similar, to that of the controls (Fig. 4).

Influence of tamoxifen on estrogen metabolite-induced cell proliferation

The treatment with tamoxifen 10^{-7} M strongly reduced the effects of the hydroxylated estrogens on cell proliferation. In fact, even after 72 hours of cell culture, there was no difference in the cell number for the majority of metabolites and concentrations compared to untreated control. Interestingly, 2OH-E1 still induced proliferative effect at the concentration of 10^{-10} M (+52% *vs.* cnt. $p<0.05$) (Fig. 5B). 2OH-E2 still showed a strong proliferative effect, with increase of 46% in the cell number at the concentration of 10^{-9}M and of 56% at the concentration of 10^{-10} M ($p<0.01$ and $p<0.05$ respectively) (Figs. 5E-F and Table II).

Discussion

Our results show for the first time that all the major downstream estrogen metabolites exert modulatory effects on monocyte proliferation and might therefore be considered active players also in the synovial tissue inflammatory reaction (18).

Interestingly, most of the hydroxy-estrogen metabolites exert a dose-dependent action. The estrone-metabolites 4OH-E1 and 2OH-E1 showed a significantly increase of cell growth at lower concentrations. Opposite effects were observed for 16OH-E2 and 4OH-E2. These metabolites, in fact, significantly enhanced cell proliferation at higher concentrations and induced a reduction of cell number at lower doses. Similarly, previous studies showed that also 17β-estradiol (E2) has dose-dependent effects. In fact at physiological concentrations (10^{-10} M), E2 increases cytokine production in macrophages, whereas pharmacological concentrations of E2 decrease pro-inflammatory cytokine levels (19, 20). As a matter of

Table II. Summary of the observed cell counts (72 hours of culture) on addition of both Tamoxifen 10^{-7} M and increasing concentrations of estrogen metabolites and the relative values (RVs) compared to the control counts. All values are to be intended x10^{6}. Each result represents the mean value of three independent experiments.
$^{*}p<0.05$.

	estrogen metabolite concentrations	
Metabolites control count	10^{-10} M Counts and RVs	10^{-9} M Counts and RVs
20H-E1	494	365
323	1.53*	1.13
20H-E2	506	472
323	1.56*	1.46*
40H-E1	246	202
323	0.76	0.62
40H-E2	305	268
323	0.94	0.83
16OH-E1	394	279
323	1.22	0.86
16OH-E2	230	276
323	0.71	0.85

fact, 16-hydroxyestrone and 4-hydroxyestradiol have been found significantly increased in RA synovial fluid and from previous results it is known that both metabolites induce monocyte cell proliferation at high concentrations (8). Therefore, it is now evident and demonstrated that an altered metabolism of 17β-estradiol could significantly influence the cell proliferative response, at least on cultured monocytes.

Furthermore, it is of interest that 2OH-E2 shows a constant proliferative effect on activated monocytes. This important finding confirms the 2OH-E2-effects already observed in previous studies on MCF-7 cells and on anterior pituitary cells, and might reinforce the observed mitogenic action on human monocytes (then differentiated in macrophages) here observed (21, 22). This proliferative effect of 2OH-E2, in contrast to the other metabolites we investigated, is not inhibited by tamoxifen, showing that this hydroxy-estrogen does not act through the estrogen receptors. Therefore, it will be of interest to further investigate the effects of this metabolite on activated monocytes.

Conclusions

The present investigation clearly suggests that hydroxylated estrogen metabolites should not be considered passive peripheral products of estrogen metabolism, but rather molecules directly involved in important cellular activities.

Furthermore, since hydroxylated downstream metabolites of 17β-estradiol and estrone are present in the synovial fluid/tissue and their metabolism is altered in RA patients, they might play a crucial role in synovitis by exerting proliferative effects. Finally, these results suggest perspectives for hormonal manipulation in RA and might support new therapeutical options.

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