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INFLUENCE OF RETINOIDS ON SKIN FIBROBLASTS METABOLISM *IN VITRO*

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Abstract: The most dangerous environmental factor for our skin condition is ultraviolet light radiation. Chronic exposition to ultraviolet light can induce epidermal atrophy, keratosis, depigmentation and dysplasia. In the dermis, UV light causes dramatic up-regulation of extracellular matrix-degrading enzymes. Matrix metalloproteinases (MMPs) are engaged in collagen, elastin and other extracellular matrix components degradation. In addition, to increase level of destructive enzymes, UV light has been shown to decrease collagen production. As a consequence of UV impact on skin, it shows signs of aging including loss of tone and elasticity, increased skin fragility, blood vessels weakness and wrinkles. The most dangerous effect of UV on skin is an increased risk of melanoma and other skin cancers. Retinoids are well known antiaging agents. For many years this vitamin has been used for the prevention and treatment of photoaging. Retinoids abolish cellular atypia, increase compacting of the stratum corneum and reduce skin hyperpigmentation caused by sun light. Recent evidence suggests that retinoids also play a role in the prevention of aging, because of its inhibitory effects on metalloproteinases expression. The aim of this study was to examine if all-*trans*-retinoic acid (ATRA) effects MMP-1, MMP-2, MMP-3 and MMP-14 gene expression in fibroblasts cultured *in vitro*.

Keywords: all-*trans*-retinoic acid (ATRA), matrix metalloproteinases (MMPs), fibroblasts, real time QRT-PCR, mRNA quantification, ultraviolet light radiation (UV radiation)

Vitamin A consists of a group of at least 50 structurally related chemical compounds. The term "retinoids" was introduced in 1976 to describe retinol (vitamin A) and its natural and synthetic derivatives (1). Retinoids exist as intracellular transmitters engaged in gene expression. In human body they play essential role in development, angiogenesis and vision. Additionally, they modulate cells biology and take a part in the body homeostasis (2). The most important role in the human body play natural forms of retinoids: retinol (ROL), retinyl esters (RE), retinal (RAL) and retinoic acid (RA). The most potent in exerting the biological action forms of vitamin A are all-*trans*-retinoic acid (ATRA, tretinoin) and 9-*cis*-retinoic acid (9CRA)(2).

In cosmetics, the most commonly used forms of retinoids are: retinyl palmitate and retinyl acetate. Retinyl esters, after its application on skin surface with cosmetics or medical ointments are stored in keratinocytes where they are metabolized into biologically active retinoids (1-3). Retinoids mediate their biological affects thought binding to nuclear receptors known as retinoic acid receptors (RARs) and retinoid X receptors (RXRs) (1, 2). RAR receptor possesses high affinity to all-*trans* retinoic acid and 9-cis-retinoic acid, whereas RXR binds 9- *cis*-retinoic acid (2). The RARs and RXRs always exist as dimers. The RARs exist as heterodimers complexed with RXRs, whereas the RXRs can exist as homodimers or heterodimers with RARs or variety of other nuclear receptors (4). Nuclear retinoid receptors bind to specific regulatory sequences in targeted genes promoter regions called retinoic acid responsive elements (RARE) and retinoid X responsive elements (RXRE) (2, 5, 6).

In the skin, retinoids are involved in keratinocyte cells differentiation, reduce epidermis cells adhesion and facilitate corneocyte exfoliation from the surface of epidermis. In the dermis retinoids regulate fibroblast proliferation, induce angiogenesis and play essential role in collagen and elastin fibrils synthesis. Because retinoids are able to inhibit arachidonic acid transformation, they possess antiinflammatory properties as well. Retinoids regulate melanogenesis and are involved in melanin granules distribution to kertinocytes. Because retinoids normalize exfoliation processes of sebaceous glands, they have anticomedogenic properties as well (1, 2, 6, 7). Recent evidence suggests additionally that topically applied on skin, retinoids may

also regulate extracellular metalloproteinases gene expression (1). Metalloproteinases play crucial role in degradation and remodeling of dermis extracellular matrix components not only during wound healing but also during inflammation, skin aging and photoaging, as well as in tumor invasion (8, 9). The ability to regulate metalloproteinases expression would be useful in inhibition of these processes.

MATERIALS AND METHODS

Cell culture

The HFF-2 (*Human Foreskin Fibroblasts*; ATCC) and NHDF (*Normal Human Dermal Fibroblasts*, Adult; Clonetics™) cells were maintained at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 1% penicillin – streptomycin solution in atmosphere of 95% humidity and 5% CO_2. At confluence, cells were routinely passaged using 1% trypsin with EDTA. Split ratio was 1 : 4. In the study cells from passage 4 to 6 were used. All-*trans* retinoic acid (ATRA), DMEM, penicillin – streptomycin solution, Dulbecco's phosphate buffered saline and trypsin with EDTA were purchased from Sigma Aldrich (St. Louis, USA). Fetal bovine serum (FBS) was from GibcoBRL (Rockville, MD, USA) and WST-1 Cell Proliferation Reagent was purchased form Roche Diagnostics GmbH (Mannheim, Germany).

Proliferation analysis of cells treated with ATRA

All-*trans* retinoic acid (ATRA) was dissolved in dimethyl sulfoxide (DMSO) as 1% stock solution and diluted in DMEM with FBS and antibiotics to 0.1%; 0.01%; 0.001%; 0.0001%; 0.00001%; and 0.000001%. NHDF and HFF-2 cells viability and proliferation treated by ATRA was assayed by spectrophotometric method with Cell Proliferation Reagent WST-1. Cells were cultured in 96-wells microplates at concentration of 5×10^3 cells/well in 100 µL cultured medium. After 24 h, when cells adhere to the bottom of wells and start growing, the medium was changed on medium supplemented with diverse concentration of ATRA. Cells were incubated with ATRA 24 h under standard culture condition (37°C, 5% CO_2 and 95% humidity). After incubation, the proliferation test was run according to the manufacturer's protocol. To each well with 100 µL DMEM, 10 µL of WST-1 test was added. After 45 min of incubation the absorbance in the cell cultures was measured ($\lambda = 450$ nm) with WALLAC Victor^2 1420 MULTILABEL COUNTER (Perkin Elmer).

Analysis of MMPS expression in HFF-2 cells treated with ATRA and UV light

For MMP-1, MMP-2, MMP-3 and MMP-14 expression analysis in HFF-2 cells cultured *in vitro*,was performed. Approximately 5×10^3 cells were seeded on Petri dishes (diameter 9 cm^2). After 24 h incubation under standard culture conditions (37°C, 5% CO_2, 95% humidity), the medium DMEM with 10% FBS and 1% pen/strep was changed on medium supplemented with 0.01%, 0.001%, or 0.0001% concentration of ATRA, respectively. A part of cells was irradiated 15 min by UV light 24 h after medium change. RNA extraction was conducted the day after cells UV irradiation. Control group of cells in this study constituted HFF-2 cells cultured in DMEM supplemented with 10% FBS and 1% pen/strep solution, in atmosphere of 95% humidity and 5% CO_2. Control cell cultures were not treated either with all-*trans* retinoic acid or with UV light.

RNA extraction

Total RNA was extracted from cell culture specimens with the use of TRIZOL® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA extracts were qualitatively checked by electrophoresis in 1.0% agarose gel stained with ethidium bromide. RNA concentration was determined spectrophotometrically using a Gene Quant II RNA/DNA Calculator (Pharmacia Biotech, USA). All RNA extracts were treated with DNase I (MBI Fermentas) according to the manufacturer's instruction.

Design of specific primers

Primers specific for MMP-1 (GenBank accession no. NM_002421), MMP-2 (GenBank accession no. NM_004530), MMP-3 (GenBank accession no. NM_002422) and MMP-14 (GenBank accession no. NM_004995) mRNAs were designed using Primer Express™ v.2.0 software (PE Applied Biosystems, USA). The primer sequences listed in Table 1 were used.

Real-time QRT-PCR assay

Transcriptional activity of MMP-1, MMP-2, MMP-3, MMP-14 genes was evaluated on the basis of copy number of mRNA related to 1 µg of total RNA by the use of real time QRT-PCR technique with a SYBR Green I chemistry (SYBR Green Quantitect RT-PCR Kit, QIAGEN, Valencia, CA, USA). Analysis was carried out using an Opticon™ DNA Engine Sequence Detector (MJ Research, USA). QRT-PCR assay was performed in triplicate

Table 1. Primers sequences used in QRT-PCR.

MMPs	Primer forward	Primer reverse
MMP-1	5'-gCTCATgAACTCggCCATTCTCTTggACT-3'	5'-CgggTAgAAgggATTTgTgCgCATgTA-3'
MMP-2	5'-TCCACTgTTggTgggAACTCA-3'	5'-TggTCgCACACCACATCTTT-3'
MMP-3	5'-AAATgCAgAAgTTCCTTggATTgg-3'	5'- TCgggATgCCAggAAAggTTCT-3
MMP 14	5'- CgCTgCCATgCAGAAgTTTTACggCTT-3'	5'-CgTAgCgCTTCCTTCgAACATTggCC-3'
β-actin	5'-TCACCCACACTgTgCCCATCTACgA -3'	5'-CAgCggAACCgCTCATTgCCAATgg-3'

Table 2. Comparison of MMPs expression in HFF-2 cultured under different conditions.

Comparison of MMPs expression in HFF-2 cultured under different conditions	MMP-1	MMP-2	MMP-3	MMP-14
C/ C+ATRA 0.01%	NS	NS	NS p = 0.02	
C/ C+ATRA 0.001%	p = 0.019	p = 0.035	p = 0.045 NS	
C/ C+ATRA 0.0001%	NS	NS	NS	NS
C/ C+UV	NS	NS	p = 0.007	NS
C+UV/ C+ATRA 0.01% + UV	NS	NS	NS	NS
C+UV/ C+ATRA 0.001% + UV	NS	p = 0.005	p = 0.034	NS
C+UV/ C+ATRA 0.0001% + UV	NS	NS	NS	NS
C+ATRA 0.01%/ C+ATRA 0.01% + UV	NS	NS	NS	p = 0.01
C+ATRA 0.001%/ C+ATRA 0.001% + UV	NS	NS	p = 0.025	NS
C+ATRA 0.0001%/ C+ATRA 0.0001% +UV	NS	NS	p = 042	NS
C+ATRA 0.01%/ C+ATRA 0.001%	NS	NS	NS	p = 0.007
C+ATRA 0.01%/ C+ATRA 0.0001%	NS	NS	NS	p = 0.006
C+ATRA 0.001%/ C+ATRA 0.0001%	NS	NS	p = 0.009	p = 0.005

Abbreviations used in the Table: C – HFF-2 cells cultured under standard conditions; C+ATRA – HFF-2 cells treated by ATRA; C+UV – HFF-2 cells cultured under standard conditions and irradiated by UV; C+ATRA+UV – HFF-2 cells treated by ATRA and irradiated by UV. NS – no statistically significant.

for each sample. The thermal profile for one-step RT-PCR was as follows: 50°C for 30 min for reverse transcription and 95°C for 15 min, 50 cycles at 94°C for 15 s, 60°C for 30 s and 72°C for 30 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and β-actin were used as internal controls in each single QRT-PCR for all samples. Moreover, the relative quantity of mRNA MMP was normalized to the respective quantity of mRNA β-actin and expressed as a ratio. RT-PCR products were separated on 8% polyacrylamide gel and visualized with silver staining. Finally, specificity of RT-PCR reaction was confirmed by determining characteristic temperature of melting for each amplimer, i.e., MMP-1: 81°C; MMP-2: 82.6°C; MMP-3: 83°C; MMP-14: 83.8°C.

Statistical analysis

The results were subjected to routine statistical analysis using the computer program Statistica PL 5.1. (Stat Soft Inc., Tulsa, OK, USA) with significance level set at $p < 0.05$. Student's-t test and Wilcoxon's test were applied where appropriate. The results are expressed as the mean ± standard error of the mean.

RESULTS

Cells viability and proliferation

In the study, NHDF and HFF-2 cells viability and proliferation treated with 0.1%, 0.01%, 0.001%, 0.0001%, 0.00001% and 0.000001% ATRA was assayed by spectrophotometric method. Average absorbances in control cells cultures (NHDF and HFF-2 cells cultured in standard medium without ATRA) measured after 24 h of cells incubation were 0.572 and 0.667, respectively ($\lambda = 450$ nm). Because all-*trans*-retinoic acid has very intensive yellow color, the absorbance of background (medium with

ATRA) was taken into account during cells proliferation assay (Figure 1). Microscopic observation of cells treated by different concentration of ATRA as well as absorbance analysis, let to choose tree concentration in medium of all-*trans*-retinoic acid (ATRA): 0.01%, 0.001%, 0.0001% that significantly influence NHDF and HFF-2 cells viability and proliferation. 0.01% ATRA significantly inhibited fibroblasts growth in cells cultures, whereas to other concentrations of all-*trans*-retinoic acid, didn't possess toxic effect on fibroblasts.

MMPS expression in HFF-2 cells treated with ATRA and UV light

In the present study we assessed MMP-1, MMP-2, MMP-3 and MMP-14 mRNAs levels using real time QRT-PCR in skin fibroblast cell culture (HFF-2) treated for 24 h with 0.01%, 0.001%, 0.0001% ATRA and moreover, cell culture treated with the same concentration of ATRA which has undergone UV radiation, The specificity of RT-PCR for the four target genes was confirmed by PAA electrophoreses, that revealed the presence of single products with desired length and on the basis of amplimers melting temperatures (Figure 2).

For normalization, the results were submitted as MMP mRNA to β-actin mRNA ratio. MMP-1, 2, 3, 14 mRNA were detected in all tested samples obtained from: fibroblasts treated with 0.01% ATRA (MMP-1 0.622 ± 0.487; MMP-2 6.944 ± 3.66; MMP-3 5.476 ± 2.722; MMP-14 0.505 ± 0.010); fibroblasts treated with 0.001% ATRA (MMP-1 0.003 ± 0.003; MMP-2 0.028 ± 0.024; MMP-3 0.015 ± 0.012; MMP-14 0.080 ± 0.029) and fibroblasts treated with 0.0001% ATRA (MMP-1 0.13 ± 0.005; MMP-2 0.057 ± 0.024; MMP-3 0.037 ± 0.010; MMP-14 0.054 ± 0.027) and control cells (fibroblasts not treated with ATRA) (MMP-1 0.012 ± 0.006; MMP-2 0.082 ± 0.034; MMP-3 0.101 ± 0.031; MMP-14 0.116 ± 0.047).

In the study, MMPs expression was compared in control cells (HFF-2 cells cultured in standard conditions) and in HFF-2 cells treated by different concentration of ATRA in culture medium. In fibroblasts treated with 0.01% ATRA, just MMP-14 was surprisingly high comparing with its expression in control cells. In fibroblasts treated with 0.001% ATRA, significantly lower transcriptional activity of MMP-1 ($p = 019$; t-test), MMP-2 ($p = 0.035$; t-test) and MMP-3 ($p = 0.045$) compared to control cells was revealed. In fibroblasts treated with 0.0001% ATRA any statistically important differences between MMPs expression compared to control cells were observed. Afterwards, MMPs expression was compared in control cells and cells irradiated by UV light. Just MMP-3 expression was statistically significantly higher in UV treated cells. Next, expression of MMPs in control cells irradiated by UV light and in cells treated by ATRA and irradiated by UV light was compared. Comparing MMPs expression in control cells treated by 0.01% ATRA and cells treated by 0.01% ATRA and irradiated by UV light, the differences in transcriptional activity of MMP-14 was revealed. In cells treated with 0.01%ATRA and UV the MMP-14 expression was lower. In cells treated 0.001% ATRA and irradiated by UV MMP-2 and MMP-3 expression was significantly higher (MMP-2 $p = 0.005$ and MMP-3 $p = 0.034$), whereas in cells treated by 0.001% and 0.0001% ATRA and irradiated by UV MMP-3 expression was significantly higher. At the end, MMPs expression in fibroblasts treated by different concentration of ATRA was compared. No statistically significant differences in MMP-1, MMP-2 and

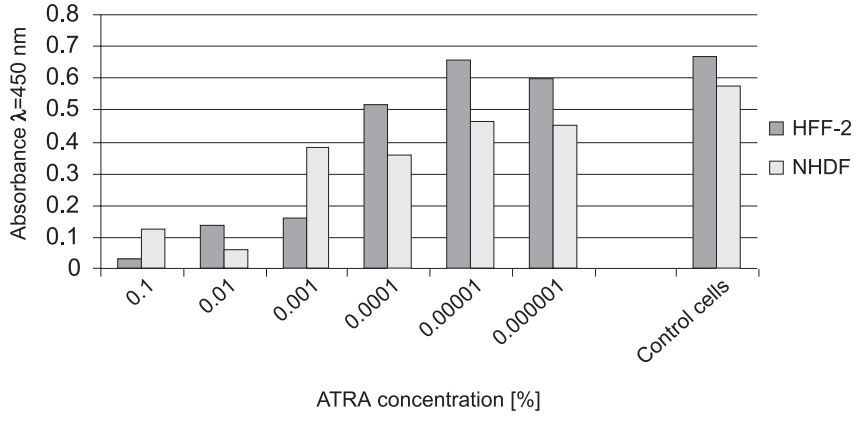

Figure 1. Dependence of absorbance at $\lambda = 450$ nm for the range of ATRA concentration.

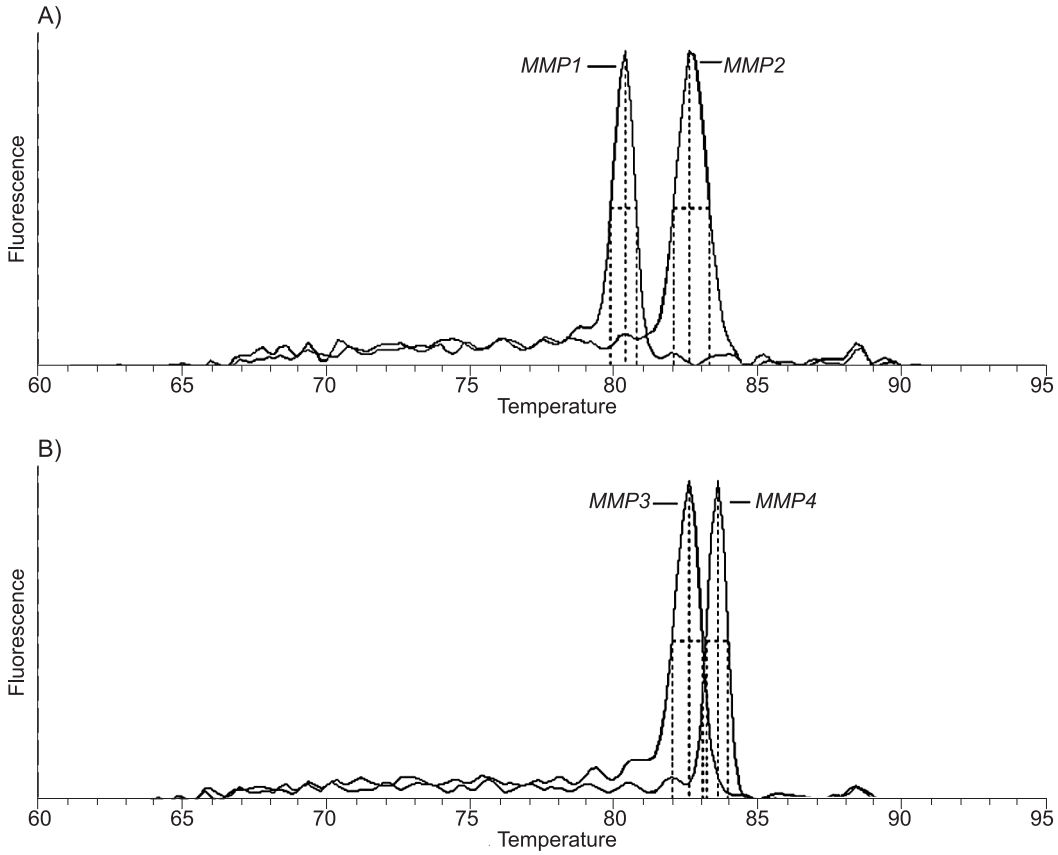

Figure 2. Melting curve analysis of the PCR products of: A) MMP-1 and MMP-2 amplimers; B) MMP-3 and MMP-14 amplimers.

MMP-3 expression were revealed in comparison with cells treated by 0.01% and 0.001% ATRA, as well as in comparison of these genes transcriptional activity in cells treated by 0.01% ATRA and 0.0001% ATRA. Whereas MMP-14 expression was significantly lower in cells treated with 0.001% ATRA and 0.0001% ATRA comparing to fibroblasts treated by 0.01%ATRA. MMP-3 expression was higher in cells treated with 0.0001% ATRA comparing to expression in cells treated with 0.001% ATRA, whereas MMP-14 expression were lower in cells treated by 0.0001%ATRA comparing to cells treated with 0.001% ATRA. A comparison of MMPs expression in HFF-2 cultured under different conditions is presented in Table 2.

DISSCUSION

Physiologically, in a healthy, youthful skin the synthesis and degradation of the extracellular matrix (ECM) are balanced. Dermal fibroblasts not only produce and organize the ECM of the dermis, but also generate active enzymes able to degrade the ECM components. Matrix metalloproteinases (MMPs) are traditionally thought to degrade the ECM in normal turnover and disease. Human skin, like all other organs, undergoes chronological aging. During senescence, the expression of MMPs increases, but expression of its naturally occurring tissue inhibitors (TIMPs) decreases. Additionally, unlike other organs, skin is in direct contact with environment agents. Exposure on these environment agents may caused skin lesion and accelerate aging process. The primary environmental factor that causes human skin aging is the sun UV irradiation. UV induces the MMPs which degrade skin connective tissue and may contribute to premature skin aging (photoaging) (10, 11).

Retinoic acid, especially all-*trans*-retinoic acid (ATRA) regulates morphogenesis, cell proliferation, differentiation, apoptosis and ECM production (12, 13). ATRA treatment stimulates collagen synthesis and reduces MMPs expression in naturally aged skin (14). These effects are mediated by nuclear receptors that are ligand-dependent transcription factors, the RARs and the RXRs, each have three distinct

receptor subtypes (α, β, γ) that are encoded by three separate genes (2, 15, 16).

In our study we investigated the cells viability and proliferation of cells treated by 0.1%, 0.01%, 0.001%, 0.0001%, 0.00001% and 0.000001% ATRA. 0.01% ATRA significantly inhibited fibroblasts growth in cells cultures, whereas other concentrations of all-*trans*-retinoic acid, did not possess cytotoxic effect on fibroblasts. Zhang et al**.** (17) described the dose- and time-dependent cytotoxic effects of ATRA which was mediated through dysfunction of mitochondria, alterations in cell cycle and induction of apoptosis. The process of retinoic acid-induced apoptosis is dependent on Bax (18) and Bcl-2 protein (19) and the nuclear transcription factor TR3 (20), transcription factor Ets1 (21), activation of p38 and the caspase cascade, but not through p53 (22).

In our study we investigated the gene expression profile of collagenase-1 (MMP-1) , gelatinase A (MMP-2), stromelysin-1 (MMP-3) and membrane type 1-matrix metalloproteinase (MMP-14) using real time QRT-PCR. The advantage of this technique is significant reproducibility and sensitivity in detection of gene expression in comparison with conventional quantitative methods. The SYBR Green – based real-time quantitative PCR procedure has been established to detect transcriptional activity and verify products by melting curve analysis. 0.01% ATRA stimulates MMP-14 expression but does not influence the expression of MMP-1, MMP-2 and MMP-3, whereas 0.001% ATRA concentration in culture medium, inhibits MMP-1, MMP-2 and MMP-3 in HHF-2 cells but does not influence MMP-14 expression in this kind of cells. The results we obtained point at a significantly lower transcriptional activity of MMP-1, MMP-2, MMP-3 ($p = 0.19$; $p = 0.035$; $p = 0.045$, respectively) in fibroblasts treated with 0.001% ATRA than in control cells. The 0.0001% concentration of ATRA in culture medium is too low to influence the examined MMPs genes expression.

The collagenase-1 (MMP1) and stromelysin-1 (MMP-3) promoters can be directly regulated by the AP-1 transcriptional activator (23), which consists of a heterodimer of c-Fos and c-Jun proteins. Dedieu and Lefebvre (24) showed the ability of retinoids to inhibit AP1 activity, and dimers c-Jun and/or c-Fos may constitute one target of retinoids for transrepression of AP1. The promoter of gelatinase A (MMP-2) contains AP-2 binding sites, but no AP-1. Similar to the MMP-2 promoter, MT1-MMP lacks an AP1 consensus binding site. Nie et al. (25) observed that an increase in MMP-2 activity induced by RA was accompanied by an increase in MMP-2 mRNA levels. This up-regulation of MMP levels by RA in chondrocytes is consistent with its effects on osteoblasts and osteosarcoma cells and opposite to its inhibitory effects on fibroblasts and endothelial cells. It seems consistent with presented results.

In the present study MMPs expression was compared in control cells and cells irradiated by UV light. UV induces the matrix metalloproteinases collagenase, 92-kD gelatinase, and stromelysin, which degrade skin connective tissue. The primary mechanism by which UV irradiation initiates molecular responses in human skin is by photochronical generation of ROS. UV irradiation activates cell surface receptors, and does ligand binding, and changes signal transduction pathways (26). ROS drive activation of MAPKs, the most important of which are ERK, JNK, and p38 kinases. ERK and JNK are important in recruiting c-Fos and c-Jun to the nucleus where they activate the transcription factor AP-1 which play an important role in the activation of MMPs (27). In our study MMP-3 expression was statistically significantly higher in UV treated cells in parallel to control cells (not UV irradiated). In spite of the fact that in this study the statistically important influence of UV on MMPs expression was noticed just in the case of MMP-3, the presence of 0.001% ATRA in cultured medium influenced the MMP-2 and MMP-3 expression in cells irradiated by UV light comparing to cells treated just by UV light. MMP-2 and MMP-3 expression was higher when the cells were irradiated by UV light and additionally treated by ATRA. It is also very interesting that UV irradiation caused a decline of MMP-14 expression in HFF-2 treated with 0.01% ATRA, comparing to cells treated just by the same concentration of ATRA. Fisher et al. (28, 29) revealed that pretreatment of human skin with ATRA inhibited UV induction of c-Jun protein and, consequently, AP-1 and MMPs productions.

In this study we revealed that all-*trans*-retinoic acid influences MMPs expression in HFF-2 cells cultured *in vitro* and that MMPs expression in cells treated with ATRA depends on its concentration in culture medium.

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