All-trans-Retinoic Acid Inhibits the Development of Mesangial Proliferative Glomerulonephritis in Interleukin-6 Transgenic Mice

Yoshihito Shima a Masayuki Iwano b Kazuyuki Yoshizaki c Toshio Tanaka a Ichiro Kawase a Norihiro Nishimoto d

aDepartment of Molecular Medicine, Osaka University Graduate School of Medicine, Osaka, bFirst Department of Internal Medicine, Nara Medical University, Kashiwara, cDepartment of Medical Science I, School of Health and Sport Sciences, Osaka University, Osaka, and dLaboratory of Immune Regulation, Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan

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Retinoic acid · Mesangial cell · Glomerulonephritis · Interleukin-6 · Transgenic mice · Vitamin A

Abstract
All-trans-retinoic acid (ATRA), a vitamin A derivative, was reported to suppress the interleukin-6 (IL-6) production and to downregulate the IL-6 receptor (IL-6R) and/or its signal transducer glycoprotein 130. We investigated the in vivo antinephritic effect of ATRA on IL-6 transgenic mice which had developed mesangial proliferative glomerulonephritis (PGN) as well as its in vitro inhibitory effect on the proliferation of rat mesangial cells. In vivo experiments on IL-6 transgenic mice showed that ATRA administration suppressed proteinuria and hematuria and reduced the IL-6 concentrations; furthermore, histological examination demonstrated that it improved PGN. In vitro experiments using rat mesangial cells demonstrated that ATRA inhibited cell growth in a dose-dependent manner within a range from 10^{-4} to 10^{-6} M. This inhibition by ATRA was partially counteracted by the addition of IL-6. RT-PCR assay results showed that ATRA also reduced IL-6R, but not the glycoprotein 130 expression in mesangial cells. These findings indicate that, by blocking of the IL-6 function, ATRA may be therapeutically effective in PGN.

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Introduction

Mesangial proliferation is a common pathological feature observed not only in primary but also in secondary glomerulonephritis [1–5]. Since it frequently progresses to renal failure despite conventional treatment with corticosteroids, there is an urgent need for the development of a new and effective therapy.

All-trans-retinoic acid (ATRA) is a vitamin A derivative currently used to treat acute promyelocytic leukemia (APL) patients, because it induces the differentiation of APL cells into mature granulocytes. Recently, retinoic acid was identified in an anti-Thy-1 model as a novel potential modulator of glomerular injury [6]. Inhibition of transforming growth factor beta 1 (TGF-ß1) by ATRA was reported to explain the reduction of extracellular matrix in the glomeruli in this model [7]. The inhibitory effect of ATRA on the mRNA expression of renin, angio-
tensinogen, and angiotensin II type 1 receptor in the rat anti-Thy-1 model has also been reported, and it could be involved in the inhibition of mesangial cell proliferation [8]. We previously reported [9] that ATRA inhibits the interleukin-6 (IL-6) dependent myeloma cell growth through the downregulation of the IL-6 receptor (IL-6R) and/or its signal transducer, glycoprotein 130 (gp 130), expression on the surface of myeloma cells as well as through the suppression of the IL-6 production from both myeloma and bone marrow stromal cells. If mesangial cell growth is stimulated by IL-6 in a manner similar to that of myeloma cells, it follows that ATRA may inhibit the proliferation of mesangial cells.

IL-6 is a pleiotropic cytokine with multiple biological activities [10], and its effect on mesangial cell proliferation has been the focus of our attention. Although the role of IL-6 as a mesangial cell growth factor remains controversial [11, 12], our hypothesis is that IL-6 functions as a stimulator for mesangial cells because of the following considerations: (1) in IL-6 transgenic mice, it was shown that proliferative glomerulonephritis (PGN) develops in the presence of high concentrations of IL-6 [13]; (2) in humans, the histological severity of PGN as established by renal biopsy and the urinary IL-6 concentrations were found to correlate in patients with IgA nephropathy [14] or lupus nephritis [15], and (3) it was also reported that PGN in IL-6 transgenic mice was prevented by the treatment with anti-IL-6R antibody [16]. Although it is still unclear whether IL-6 affects mesangial cells directly or indirectly, it is reasonable to conclude that IL-6 is involved in mesangial cell proliferation. Thus, inhibition of the IL-6 signal transduction may be therapeutically effective in PGN. However, the interaction between the therapeutic effect of ATRA and the proliferative factors for mesangial cells, especially IL-6, remained to be clarified. We, therefore, investigated the in vivo antinephritic effect of ATRA on IL-6 transgenic mice with evidence of PGN and also examined the in vitro inhibitory effect of ATRA on the proliferation of rat mesangial cells as a result of the downregulation of IL-6R.

Materials and Methods

Chemical Reagents
ATRA (Sigma Chemical, St. Louis, Mo., USA), which is insoluble in water, was dissolved in 99.5% ethanol (Nacalai Tesque, Kyoto, Japan) to make a solution of $1 \times 10^{-2} M$. The solution was then diluted with D-Val-MEM supplemented with 20% fetal calf serum and antibiotics. ATRA was used at amounts of not more than $10^{-4} M$. Two precursors of ATRA, retinol and retinal (Sigma Chemical), were used to confirm whether the observed effects were specific to ATRA or common to retinoids or lipophilic vitamins. These two compounds were dissolved in ethanol in the same manner as ATRA. For in vivo experiments, 300 mg of ATRA was dissolved in 100 ml of seed oil (Nishinseiyu, Tokyo, Japan) to make a solution of $10^{-2} M$ which was then diluted with the same oil to $10^{-3} M$.

In vivo Experiments on IL-6 Transgenic Mice
The Osaka University complies with the current NIH policy on animal welfare. Experimental procedures are designed to avoid unnecessary discomfort, pain, or injury to the animals, as recommended by the resident veterinarian and in accordance with institutional policies. IL-6 transgenic mice were kindly provided by Dr. Katsume (Chugai Pharmaceutical, Tokyo). The transgenic mouse model was made by introducing human IL-6 cDNA combined with the H-2Ld of mouse MHC class I promoter into the C57BL/6J mouse. The experiments were conducted under specific pathogen free conditions in compliance with the ethical regulations of the Institutional Animal Research Committee of the Osaka University.

Four groups of 5 mice each were used for the study. Two groups received daily ATRA dissolved in seed oil at doses of 30 or 300 µg/100 µl/mouse for 6 weeks, starting at the age of 7 weeks. The therapeutic doses were determined with reference to those used for patients with APL. The third group, the oil control group, received only the oil vehicle at 100 µl/mouse daily, and the fourth group, the control group, was given neither ATRA nor the oil vehicle. The reagent or oil was administered by means of transesophageal tubes. Urine samples were collected once a week to monitor proteinuria and hematuria by means of semiquantitative analysis with the Urotrin RL9 system (Boehringer Mannheim Yamanouchi, Tokyo). This system detects and assesses proteinuria at 300 mg/l as 1+, at 1,000 mg/l as 2+, and at 5,000 mg/l as 3+ and hematuria at about 10 RBC/l as 1+, at 50 RBC/l as 2+, and at 500 RBC/l as 3+. Retro-orbital bleeding every 2 weeks yielded blood samples from which the serum was separated. The concentrations of human IL-6 in serum were determined with a Lumipulse 1200 (Fujirebio, Tokyo) by means of the chemiluminescent enzyme immunoassay (CLEIA) [17]. On the 42nd day after the start of medication, the mice were sacrificed, and the organs including right kidney, spleen, and liver were fixed in 20% formalin. Histological examination was performed by staining with periodic acid-Schiff and hematoxylin-eosin.

Frozen sections were also prepared for immunohistochemical analysis. After a blocking procedure using Block Ace® (Dainippon Pharmaceutical, Tokyo), tissue sections were incubated for 12 h at 4°C with the following antibodies: rat antimonoclonal macrophage antibody F4/80 (Caltag Laboratories, Burlingame, Calif., USA) and rabbit antimonoclonal antibodies (Vector Laboratories, Burlingame, Calif.) used as the secondary antibodies, and horseradish peroxidase conjugated avidin D (Vector Laboratories) was used to detect their expression. Counterstaining consisted of methylene green staining.

Preparation of Mesangial Cells
Rat mesangial cells were used for the in vitro experiments because of the following considerations: (1) rat mesangial cells have been used for in vitro experiments universally and historically – the method to obtain them was established; (2) rat mesangial cells can be more easily identified by their Thy-1 antigen expression than mouse or human cells, and (3) the rat kidney is easier to operate than that of the mouse when the organs are sieved. Mesangial cells were isolated from rats with the sieving method [18]. Briefly, both kidneys were

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resected under ether anesthesia from female Sprague-Dawley rats weighing 175–200 g. Renal cortex tissues were then cut into small pieces and sieved. Samples of glomeruli that did not contain either Bowman’s capsules or tubular epithelial cells were obtained from the fractions passed through stainless steel sieves with openings of 106 μm, but not through ones with 70-μm openings. The glomeruli were cultured in D-Val-MEM supplemented with 20% fetal calf serum (Gibco Laboratories, Grand Island, N.Y., USA) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in a 5% CO2 atmosphere to obtain mesangial cells for the experiments. The isolated rat mesangial cells used for the in vitro experiments were identified by their Thy-1 antigen expression [19], and mesangial cells from the seventh passage were used for our study.

**Cell Proliferation Assay**

The in vitro effect of ATRA on the proliferation of rat mesangial cells was examined by means of [3H]-thymidine – [3H]-TdR (DuPont, Wilmington, Del., USA) – incorporation as described elsewhere [8]. Rat mesangial cells were cultured at a density of 1 × 103/well in D-Val-MEM supplemented with 20% fetal calf serum in 96-well flat-bottom plates for 24 h. After confirmation of cell adhesion to the bottom of the plates, 100 μl of the medium was removed from each well followed by the addition of 2 × 10−10 to 2 × 10−4 M of ATRA or 2 × 10−6 M of retinol or retinal (100 μl each) to each well. Ethanol, used as a solvent of ATRA, was added to the medium of the controls at the same concentrations as those of ATRA. After incubation for 48 h, 18.5 kBq/well of [3H]-TdR (370 GBq/mmol) was added, followed by cell culture for another 48 h. The radioactivity of the incorporated [3H]-TdR was measured with a scintillation counter. To determine the relationship between the effect of IL-6 and that of ATRA on the growth of mesangial cells, rat mesangial cells were cultured with serial concentrations of both IL-6 and ATRA. First, rat mesangial cells (5 × 104/well) were seeded into 96-well plates, and after a 72-hour incubation, IL-6 was added (1 or 100 ng/ml). ATRA was also added at the same time (10−4 or 10−8 M). The proliferation of the cells was evaluated with the [3H]-TdR method described above.

**RT-PCR Assay for IL-6 R and gp130**

The RT-PCR assay was initiated by coamplifying D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA for semiquantitation of IL-6 R and gp130 mRNA in mesangial cells. After incubation with 10−4 M of ATRA for 6 h, total RNA was extracted in RNA zol (Biotex Laboratories, Houston, Tex., USA). Total RNA (1 μg) was then reverse transcribed into cDNA with a first-strand cDNA synthesis kit (Amersham Biosciences, Chalfont St. Giles, UK). To the resultant reaction mixture, 100 μM of random hexamer, 1 mM each of dATP, dCTP, dTTP, and dGTP, and 1 U/μl of RNase inhibitor (Amersham Biosciences) were added. The reagents were incubated at 42°C for 1 h and then heated at 95°C for 5 min to denature the RNA-cDNA hybrid and to inactivate reverse transcriptase. PCR was performed in a 100-μl solution containing cDNA, a reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM MgCl2), dNTPs (0.2 mM), 2.5 U Taq DNA polymerase (Amersham, Little Chalfont, UK), and 0.2 μM of each of the two primers. The nucleotide sequences were: upstream primer for IL-6 R 5'-CGTGAAGG-CAATGACACTG-3' (from nucleotide 591 to 612 of the cDNA); and downstream primer for IL-6 R 5'-GCGGAAGGGCGGAGTACCT-3' (from nucleotide 423 to 442 of the cDNA). The PCR cycle consisted of denaturation at 94°C for 1 min, primer annealing at 65°C for 30 s, and extension at 72°C for 1 min. After amplification (35 cycles for IL-6 R and 25 cycles for gp130 and GAPDH), the PCR products were poured onto 2% agarose gel (FMC Bioproducts, Rockland, Mass., USA). The concentration intensity of ethidium bromide was then quantified with a densitometer (Atto, Tokyo) to measure the ratio of IL-6 R or gp130 cDNA to GAPDH cDNA.

**Results**

**Urinalysis of IL-6 Transgenic Mice**

Figure 1 shows the time course of the results of the urinalysis as values of the semiquantitated scores for each group. Proteinuria and hematuria were observed in both...
Fig. 2. Histological features of the kidneys of IL-6 transgenic mice treated with oil vehicle alone or ATRA. The IL-6 transgenic mice treated with the oil vehicle alone showed mesangial cell proliferation and capillary occlusion (A). In contrast, only minor mesangial cell proliferation was observed in the transgenic mice treated with 300 μg/day of ATRA (B). A × 200, B × 400. The magnification of A is lower than that of B because of glomerular expansion in A.

Pathological Findings
For macroscopic studies, the kidneys were analyzed at the time of sacrifice after 42 days of ATRA administration to determine its effect on the kidney size. Although ATRA has been reported to inhibit the compensatory hypertrophy of the kidney after unilateral removal [20], there was no substantial difference in size among the two groups of ATRA-treated mice, the oil-treated controls, and the untreated controls. The splenic weight was also measured to determine whether ATRA could eliminate splenomegaly in IL-6 transgenic mice. There was a tendency for splenic weight to decrease in the ATRA-treated groups, although the difference between the ATRA-treated groups and the control groups was not statistically significant because of the large individual variations (data not shown).

Microscopic analysis showed mesangial cell proliferation with occlusion of the glomerular capillaries both in the oil-treated control mice and in the untreated control mice (fig. 2). These images suggested a reduction of blood flow in the glomeruli, though functional parameters, particularly the creatinine clearance, were not measured. In contrast, the normal morphology of the capillaries was retained with only a mild proliferation of mesangial cells in the mice treated with 300 μg of ATRA. Next, the number of nuclei in glomeruli was examined for a quantitative
assessment of a pathological increase in mesangial cells. For each section, sets of 10 glomeruli were randomly selected from four sites in the renal cortex, namely the superficial and deep layers in the upper pole and the superficial and deep layers in the lower pole. There was a tendency for the number of mesangial cells to decrease in the mice treated with ATRA, especially in the 300-µg group, although no statistically significant difference was observed because of the large individual variations (fig. 3).

Because IL-6 is a potent stimulator for macrophages, it may also stimulate the infiltration of macrophages into glomeruli. However, the antimouse macrophage marker antibody F4/80 stained few cells in the glomeruli (fig. 4A). Macrophage infiltration into glomeruli appeared minimum. These results indicate that ATRA produced an inhibition of mesangial cell proliferation in vivo. Laminin and collagen type IV were stained in the glomeruli of both ATRA-treated and untreated mice (fig. 4C–F). The extent of the stained area in the glomeruli depended upon the extent of cell proliferation.

With respect to adverse reactions associated with ATRA treatment, we were interested in hepatic abnormalities, because the liver is a major organ for storage of vitamin A and hence a site of possible degeneration by overdosing [21]. However, we could not find any collapse of the hepatic cords, infiltration of inflammatory cells, or destruction of limiting plates of the liver even in the 300-µg group. This indicates that this dosage of ATRA did not cause liver damage, although it should be noted that the hepatic function in terms of blood enzyme levels was not assessed.

Dermatitis occurred in the form of loss of hair, desquamation, or partial bleeding in the extremities or the abdomen of the mice given 300 µg of ATRA. Since these changes in appearance were not observed in mice given 30 µg of ATRA, the skin troubles may have been caused by the higher concentration of ATRA in the sera.

**CLEIA Assay of Serum IL-6 Levels in IL-6 Transgenic Mice Treated with ATRA**

The serum IL-6 concentrations were determined with the CLEIA assay. The serum IL-6 concentrations increased over time in both the untreated and the oil-treated control group, but there was no significant difference between the two groups. Administration of ATRA inhibited the elevation of serum IL-6 (fig. 5) to a greater extent in the 300-µg group than in the 30-µg group, thus indicating dose dependency.

**In vitro Inhibitory Effect of ATRA on the Proliferation of Rat Mesangial Cells**

Figure 6A shows the changes in rat mesangial cell proliferation induced by ATRA and assessed in terms of [3H]-TdR incorporation. ATRA inhibited the mesangial cell proliferation in a dose-dependent manner from 10^{-6} to 10^{-4} M, while ATRA at 10^{-10} M did not exert any significant inhibitory effect. The average count of [3H]-TdR was reduced to 3.2%, with an increase in ATRA from 10^{-10} to 10^{-4} M. No significant inhibition was observed in response to 10^{-6} M of either retinol or retinal, both of which are derivatives of vitamin A, in comparison with ethanol controls.
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Fig. 5. Suppressive effect of ATRA on the serum IL-6 concentrations in IL-6 transgenic mice. The IL-6 transgenic mice showed an increase over time in the mean levels of the serum IL-6 concentrations. The levels were reduced in ATRA-treated transgenic mice in a dose-dependent manner. The mice were given daily doses of ATRA in seed oil of 300 \( \mu \text{g/mouse} \) (○) and 30 \( \mu \text{g/mouse} \) (●) or oil vehicle alone (□). ○ = Untreated mice. Data are expressed as mean values ± SD. The asterisk (*) indicates a significant difference as compared with the oil-treated control group. The t test was used for statistical analysis.

Fig. 6. Inhibitory effect of ATRA on the growth of mesangial cells in vitro. The cell proliferation assay demonstrated a concentration-dependent inhibition of rat mesangial cell growth by ATRA (A). Cells were cultured with 1% ethanol, 10\(^{-6}\) M retinal or retinol, or varying amounts of ATRA (10\(^{-12}\) to 10\(^{-4}\) M). DNA synthesis was measured by means of [\( ^{3}\text{H} \)]-TdR incorporation after a 4-day culture. Mean values ± SD are shown. * \( p < 0.001 \) and ** \( p < 0.0001 \), significantly different from results with 1% ethanol. IL-6 partially counteracted the inhibitory effect of ATRA in mesangial cell growth (B). + \( p < 0.01 \) and ++ \( p < 0.001 \).

Furthermore, the inhibition of mesangial cell growth by 10\(^{-8}\) M of ATRA was partially counteracted by the addition of IL-6, although IL-6 could not reactivate the cell growth inhibited by 10\(^{-4}\) M of ATRA. A slight inhibition of cell growth in response to 100 ng/ml of IL-6 and 1% ethanol was due to contact inhibition caused by overgrowth (fig. 6B).

RT-PCR Assay for IL-6R

The RT-PCR assay was used to determine the effect of ATRA on IL-6R and gp130 expression. The results showed that coculture with ATRA reduced the expression of IL-6R in rat mesangial cells (fig. 7). Measurements performed with a densitometer showed a ratio of IL-6R to GAPDH of 1.20 ± 0.26 for the ethanol-treated cells and 0.16 ± 0.02 (87% reduction) for the ATRA-treated cells.

It was thus demonstrated that ATRA downregulated the IL-6R but not the gp130 expression in rat mesangial cells.

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Discussion

Our in vivo experiments using IL-6 transgenic mice showed that ATRA administration delayed the onset of both proteinuria and hematuria and reduced their severity. In addition, histological examination demonstrated an inhibition of mesangial cell growth. No histological evidence of either nodular lesions or crescent formation was observed in the glomeruli of untreated IL-6 transgenic mice, nor was infiltration of macrophages into glomeruli. Therefore, the antinephritic effect of ATRA may be realized through the inhibition of mesangial cell growth. This hypothesis was supported by the fact that ATRA inhibited mesangial cell growth in a dose-dependent manner in vitro.

In the study presented here, we used IL-6 transgenic mice to clarify how ATRA affects nephritis associated with an elevation of plasma IL-6. The reduction in serum IL-6 concentrations in mice treated with ATRA may contribute to growth inhibition. However, the serum IL-6 concentrations of ATRA-treated IL-6 transgenic mice were still higher than those of normal mice, even though the increase in serum IL-6 was eliminated by ATRA administration. Therefore, the suppression of serum IL-6 alone does not seem to be sufficient to explain the mechanism of the therapeutic effect. Besides, the underlying mechanism of IL-6 regulation seen in our study may differ from that in patients, because mouse MHC was used as a promoter in this strain of mice, and MHC itself might be influenced by retinoic acid. A study using human embryonic carcinoma cells has demonstrated that retinoic acid increased the level of MHC class I mRNA [22]. If such an effect had been manifested in the mice used in our study, the serum IL-6 concentrations should have increased, while in fact they were reduced. Thus, the mechanism by which ATRA reduces the IL-6 production in this IL-6 transgenic mouse model remains to be clarified. Another point that needs clarification is whether ATRA administration reduces the IL-6 production in vivo in humans for possible clinical application.

In our in vitro experiments, the inhibition of rat mesangial cell proliferation was considered to be a specific effect of ATRA, not shared by the retinoids at the concentrations tested, because neither retinol nor retinal inhibited mesangial cell growth. Since the peak plasma concentration of ATRA was found to be $10^{-6} M$ in APL patients receiving differentiation induction therapy with ATRA [23], the concentrations of ATRA which resulted in an inhibition of mesangial cell growth in vitro in our study are within the range of feasibility for ATRA administration for the treatment of patients. Furthermore, ATRA reduced the expression of IL-6R, but not that of gp130. It is possible that the growth-inhibitory effect of ATRA on mesangial cells is realized through the downregulation of IL-6R, as observed in myeloma cells [9]. In addition, the inhibitory effect of ATRA on mesangial cell growth was counteracted by the addition of IL-6. This finding further supports our hypothesis that ATRA inhibits mesangial cell growth via blocking of the IL-6 function.

There are other cytokines than IL-6 that may affect mesangial cells. It has been reported that retinoic acid reduces the expression of TGF-β and TGF receptor II in anti-Thy1.1 nephritis rats [7]. TGF-β is regarded as a factor in the accumulation of mesangial matrix, but its effect on the proliferation of mesangial cells is subject of considerable controversy [24, 25]. ATRA inhibited the proliferation of mesangial cells in vitro in our study, and this inhibition was affected by the concentration of IL-6 in the media. ATRA thus may exert its antinephritic effect through not only TGF-β, but also suppression of IL-6.

It has been reported that retinoic acid inhibits the proliferation of rat synovial cells in vitro, suggesting that this retinoid may antagonize the actions of epidermal growth factor and interferon gamma [26]. However, since interferon gamma has been reported to inhibit the proliferation of mesangial cells [27], it is unlikely that the inhibitory effect of ATRA on mesangial cell proliferation is caused by an antagonism to interferon gamma. Heparin-binding epidermal-growth-factor-like growth factor can also promote mesangial cell proliferation [28], but whether ATRA has any such effect is not known at present. Platelet-derived growth factor (PDGF) is the other candidate which is influenced by ATRA. ATRA treatment has been shown to inhibit mesangial cell proliferation in a rat Thy-1 model, in which mesangial cell proliferation was PDGF dependent. Retinoic acid, however, is known to enhance the mitogenicity of PDGF in psoriatic fibroblasts [29, 30], so that the involvement of PDGF in this process is still unclear.

Dermatitis was the only adverse reaction to ATRA administration in our study. This adverse reaction is sometimes observed in APL patients who are treated with ATRA. Retinoid receptors are known to be present in keratinocytes in the skin [31, 32], and retinoids have been used in the treatment of psoriasis vulgaris. It is, therefore, thought that retinoids may induce an excessive differentiation in normal keratinocytes, causing desquamation and excoriation. However, the retinoic acid receptors (RARs) in the dermal tissue are reported to be different from those in the kidney [32]. So far, three kinds of receptor

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subtypes have been identified: RAR-α and RAR-β, which are dominantly expressed in the kidney, and RAR-γ which is dominant in dermal tissue. It is expected that the development of retinoids with a minimal affinity for RAR-γ can eliminate this adverse reaction.

Our findings lead us to conclude that ATRA may have the quality as a candidate for a new therapeutic drug for PGN.

References