Effects of a Novel Synthetic Vitamin A-Like Compound (a Polyprenoic Acid Derivative, E-5166) on Neutrophil- Derived Reactive Oxygen Species

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Summary In order to elucidate the mechanisms of anti-inflammatory activity of a new synthetic vitamin A-like compound (polyprenoic acid derivative, E-5166), the anti-oxidant effect of the agent was evaluated by examining the levels of reactive oxygen species both in the zymosan-stimulated neutrophil system and in the cell-free, xanthine-xanthine oxidase system. E-5166 inhibited the generation of hydroxyl radicals but had little effect on superoxide, hydrogen peroxide, or chemiluminescence when used at therapeutic concentrations. The favorable effects of E-5166 on several inflammatory skin disorders are discussed in connection with the anti-oxidant activity of the drug.

Key Words: E-5166, reactive oxygen species, neutrophils, anti-inflammatory effect

Vitamin A and its synthetic analogs (retinoids) have been demonstrated to affect cellular differentiation and proliferation with resultant anti-neoplastic effects. In the field of dermatology, retinoids have been used for the treatment of not only keratinizing disorders but also several cutaneous inflammations such as pustular psoriasis and cystic acne, where neutrophils play an important role. These effects have been attributed to the anti-inflammatory action by retinoids [1, 2], however, the exact mechanism by which retinoids work in the inflammatory process still remains unclear.

Recently we reported that the anti-inflammatory effects of retinoids can be partly explained by their inhibition of reactive oxygen species (ROS) generation by neutrophils [3]. In the present investigation, effects of a new synthetic vitamin

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A-like compound, E-5166, on ROS levels were examined and compared with those of tretinoin (all-trans-retinoic acid).

MATERIALS AND METHODS

Chemicals. Tretinoin (all-trans-retinoic acid) was purchased from Sigma Chemical Co. (St. Louis, MO). E-5166 was a generous gift from Eisai Co., Ltd., Tokyo. All other chemicals were analytical reagent grade.

Preparation of neutrophils for ROS assays. Human neutrophils were prepared from heparinized peripheral venous blood according to a previously described method [4]. After centrifugation of the blood over a Ficoll-Hypaque gradient, the cell pellet containing neutrophils and erythrocytes was washed with saline solution and resuspended in plasma containing dextran 170 (molecular mass, 170,000 daltons) at a final concentration of 1%. The neutrophils were recovered after sedimentation at unit gravity, and the few contaminating erythrocytes were lysed by treating the preparation with 0.876% NH_4Cl . The neutrophils were then resuspended in Krebs-Ringer phosphate buffer (KRP) [5] containing 5 mM glucose and 1 mg/ml gelatin for the ROS assays. Gelatin was added in order to prevent the neutrophils from adhering to the test tubes. Gelatin was excluded from the medium used in the $\text{OH}^\cdot$ generation assay on account of its inhibitory effect.

ROS generation assays. Opsonized zymosan (Sigma) was freshly prepared before each experiment by incubating 11 mg of zymosan with 1 ml of autologous serum for 30 min at 37°C. The formation of O_2^- was determined according to the method of Johnston and Lehmeyer [6]. Neutrophils (4×10^6) were preincubated at 37°C for 10 min with opsonized zymosan at 1 mg/ml. After the addition of 0.1 mM ferricytochrome *c* (type III; Sigma), the neutrophils were incubated for a further 30 min at 37°C. Immediately after the neutrophils and opsonized zymosan had been sedimented by centrifugation, the supernatants were assayed for superoxide dismutase (SOD) inhibitable ferricytochrome *c* reduction by measuring their absorbance at 550 nm with a spectrophotometer (Hitachi, Tokyo). In order to confirm the specificity of the O_2^- assay, SOD was added at a concentration of 100 units/ml, and the inhibition of cytochrome *c* reduction by O_2^- was measured.

The generation of H_2O_2 was measured by quantitating the decrease in the fluorescence intensity of scopoletin (Sigma) resulting from its peroxidase-mediated oxidation by H_2O_2 [7]. After the incubation of 2.5×10^6 neutrophils in KRP with 1 mg/ml of opsonized zymosan for 10 min at room temperature, 0.1 ml of 50 mM scopoletin in KRP and 0.1 ml of 1 mg/ml horseradish peroxidase (type II; Sigma) in phosphate-buffered saline (PBS) were added. The rate of decrease of the fluorescence intensity of the scopoletin over a 30-min period was quantitated using a fluorescence spectrophotometer (Hitachi). Excitation and emission wavelength are 360 and 460 nm, respectively.

The generation of $\text{OH}^\cdot$ was quantitated from the amount of ethylene gas formed by the reaction of α -ketomethiol butyric acid (KMB; Sigma) with $\text{OH}^\cdot$.

generated by neutrophils [8]. Neutrophils (2×10^6) in 2 ml KRP were preincubated with 1 mM KMB for 5 min at 37°C. After (1 mg/ml) opsonized zymosan had been added, the incubation was continued for a further 10 min. The amount of ethylene gas formed was assayed at 10, 20, and 30 min using a gas chromatograph (Hitachi), and the total of these measurements served as the $\text{OH}^\cdot$ value.

Chemiluminescence was measured in a scintillation spectrometer (Packard IL) according to the method of Allen and Loose [9] with slight modifications. Neutrophils (5×10^6) in 3 ml of colorless Hanks' solution containing gelatin were incubated for 10 min at 37°C with opsonized zymosan in the absence of luminol. The chemiluminescence was monitored using a spectrometer operated in the out-of-coincidence summation mode. All procedures were performed in the dark.

Effects of E-5166 and tretinoin on the generation of ROS by neutrophils. The agents were first dissolved in dimethylsulfoxide (DMSO), and this solution was then added to the neutrophil-containing medium of each ROS assay system to make final concentrations of 1, 10, and 100 μM . The final concentration of DMSO was 0.1%. The same volume of DMSO was added to the control medium. Since DMSO is a potent radical scavenger and the generation of ROS in the controls was reduced by the addition of DMSO, the effect of the agent on ROS generation was calculated by using the control values after the addition of DMSO.

Effects of E-5166 and tretinoin on ROS generation in the xanthine-xanthine oxidase system. In separate experiments, the effects of the agents on ROS generation were investigated in the xanthine-xanthine oxidase system. Instead of adding neutrophils and opsonized zymosan, 0.1 ml of hypoxanthine solution (13.5 mg in 50 ml physiological saline) and 0.05 ml of 50 mM ethylenediaminetetraacetate (EDTA) were diluted in 2 ml KRP (pH 7.2–7.4). To generate O_2^- , 0.1 ml of 0.12 unit/ml dialyzed xanthine oxidase was added, and the amount of each ROS was determined as already described.

RESULTS

As shown in Fig. 1, tretinoin reduced the O_2^- formation dose-dependently in both cellular and cell-free ROS generation systems. However, no significant effect was observed with E-5166 in either neutrophil or xanthine-xanthine oxidase system.

The level of H_2O_2 was also decreased by tretinoin in both assay systems in a dose-related way (Fig. 2). Only the highest concentration of E-5166 inhibited the H_2O_2 generation by neutrophils, and no significant reduction in H_2O_2 level was noticed in a cell-free system by E-5166 at any concentration tested.

As demonstrated in Fig. 3, $\text{OH}^\cdot$ levels were again reduced significantly in a dose-dependent manner by tretinoin in both ROS producing systems. On the contrary, E-5166 significantly inhibited the neutrophil-derived $\text{OH}^\cdot$ generation dose-dependently without affecting $\text{OH}^\cdot$ levels in the xanthine-xanthine oxidase system. Chemiluminescence was affected by neither E-5166 nor tretinoin (Fig. 4).

Fig. 1. Effects of E-5166 and tretinoin on O_2^- levels. Each point denotes the mean of triplicate assays. Bars indicate SD. Means $\pm$ SD are given. Tretinoin: ○, neutrophil system; ●, cell-free system. E-5166: △, neutrophil system; ▲, cell-free system.

Fig. 2. Effects of E-5166 and tretinoin on H_2O_2 levels. For symbols, see the legend to Fig. 1.

DISCUSSION

E-5166 is a new synthetic vitamin A-like compound originally described by Muto and Omori [10] and Muto *et al.* [11] and has a strong *in vitro* binding affinity to intracellular binding proteins for acidic retinoids. It is clinically effective for various inflammatory skin diseases as well as hyperproliferative and dyskeratotic skin diseases. However, the exact mechanism of anti-inflammatory activity of

Fig. 3. Effects of E-5166 and tretinoin on $\text{OH}^\cdot$ levels. For symbols, see the legend to Fig. 1.

Fig. 4. Effects of E-5166 and tretinoin on chemiluminescence. For symbols, see the legend to Fig. 1.

E-5166 still remains to be studied. E-5166 is reported to inhibit the epidermal growth factor-stimulated arachidonic acid release through inhibition of the epidermal growth factor-stimulated phosphatidylinositol turn over, and thus this compound may have profound effects on phospholipid metabolism [12]. Previously we have reported anti-oxidant effects of retinoids and discussed a possible mechanism by which retinoids may scavenge ROS to exert their clinical effects on several cutaneous inflammatory disorders [3]. Since neutrophil-derived oxygen metabolites released at sites of inflammation induce tissue damage there [13], it is reasonable to speculate

that the drugs with anti-oxidant activity may work as anti-inflammatory agents. The present study demonstrates that E-5166 inhibits $\text{OH}^\cdot$ generation in neutrophil system at the therapeutic levels and this inhibitory effect of E-5166 may partly account for its clinical effectiveness against inflammatory skin diseases.

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